THERMAL INACTIVATION OF *Escherichia coli* K-12 AND BACTERIOPHAGE LAMBDA AT 52°C AND RECOVERY

ABSTRACT

THESIS SUBMITTED FOR THE DEGREE OF
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ABSTRACT

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The effect of 52°C heat treatment to Escherichia coli K-12 and bacteriophage λ were studied. The essential data can be summerized as under:

A. Effect of heat treatment on E.coli K-12.

1. The recA, lex and polA mutants of E.coli K-12 exposed to 52°C in Tris-Mg buffer (0.01M, pH 8.0) were more sensitive to heat than the wild-type strain, and uvrA and recB mutants.

2. Survival of bacteria was influenced by composition of the pre- and post-treatment growth medium. When heated cells were incubated in tryptone broth (RM) or minimal medium (MM) for 120 min before plating on tryptone agar survival depended on the composition of pre-treatment and post-treatment incubation media. Recovery was maximum when heated bacteria were held in RM for 120 min. This is "Liquid-holding recovery" of heated cells observed in broth but not if plated directly on tryptone agar.

3. During liquid holding in tryptone broth, recA and polA mutants also recovered but a lex mutant did not.

4. As a result of heating, the sensitivity of bacteria to ultraviolet radiation, mitomycin C and to plating on high

salt medium was enhanced. After incubation for 2 hr in tryptone broth at 37°C, the bacteria regained their normal resistance to UV, mitomycin C and tolerance to high salt medium.

5. Recovery of viability required RNA and protein syntheses. Addition of rifampicin and chloramphenicol in the recovery medium interfered with recovery. Rifampicin inhibited recovery of mitomycin C resistance significantly and to large extent, recovery of plating on high salt medium. Chloramphenicol, on the other hand, affected to some extent recovery of high salt tolerance and resistance to mitomycin C. Recovery of UV resistance did not require protein synthesis.

6. Heat treatment to bacteria resulted in the appearance of single strand breaks in the DNA. These breaks were repairable in the recovery medium at 37°C.

7. Less DNA was degraded in all the heated bacterial strains compared to unheated cells. This observation was made with all the bacterial strains irrespective of their genetic marker.

8. Heat treatment caused inhibition of DNA synthesis. The degree of inhibition was more in lex strains than in the wild-type bacteria. During liquid holding, recovery of DNA synthesis was observed with w.t. bacteria whereas recovery was poor with lex strains

9. RNA synthesis was also affected by heat treatment. In wild-type bacteria, RNA synthesis resumed after 30 min if incubated in recovery medium whereas it remained inhibited upto 90 min in lex strain.

10. RNA degration was observed with heated bacteria during incubation in recovery medium. The extent of degradation was more with the lex mutant than the wild-type strain.

11. During the 30 min of heat treatment to bacteria in Tris-Mg buffer, release of RNA precursors upto the extent of 4% of the total RNA was observed. Most of the leakage occurred during 10 min of exposure.

12. Leakage of proteins during heat treatment and degradation of proteins during liquid holding were not observed.

13. Permeability of E.coli to ^{3}H-actinomycin D increased with duration of heat treatment suggesting alteration of cell membrane. Liquid holding restored normal permeability of cell membrane.

14. Heat treatment to bacteria was found to be mutagenic. The frequency of ampicillin- and rifampicin-resistant colonies were measured. Mutations were less when bacteria were held in tryptone broth.

B. Effect of heat treatment on λ.

1. Extracellular heating of phage as free particles at 52°C upto 60 min had no effect on plaque forming units (PFU).

2. Intracellular heating resulted in loss of PFU. λ-*polA* complex was most sensitive to heat specially at short periods of heat treatment. Other mutations in bacteria, such as *recA*, *lex* and *uvrA* also affected survival of PFU of λ.

3. λ*red* phage was more sensitive as compared to *red*$^+$. The *red*$^+$ and *recA*$^+$ functions seem have complementary effect specially at short exposures of heat treatment.

4. Contrary to heated *E.coli*, liquid holding recovery of PFU was not observed when *E.coli* complex was held in recovery medium. An assay of β-galactosidase and synthesis of mRNA revealed that no transcription occurred in infected bacteria. On the other hand, in uninfected bacteria, these activities normally occurred. This suggested that recovery of PFU in recovery medium was not possible because infected bacteria could not recover.

5. The inactivation of PUF of λ depended very much on the state of the host. When w.t., *recA*, *polA* hosts were heated and infected with unheated λ, survival of PFU of λ declined with increase in heating of bacteria.

6. Heat treatment was not mutagenic to phage λ, no matter how λ was exposed to 52°C, i.e. extracellularly or intracellularly. Heating of bacteria alone did not enhance mutation frequency.

C. Purification of phage λ by gel filtration.

A method for purification of phage λ by gel filtration has been developed. The crude lysate of phage is subjected to nucleases and polyethylene glycol treatments prior to loading on Sephadex G-100 column. The purified phage is eluted in the void volume. The method is rapid, efficient and relatively inexpensive. The technique can also be used for large scale purification of phage λ .

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This is to certify that the thesis entitled "Thermal inactivation of Escherichia coli K-12 and bacteriophage lambda at 52°C and recovery", is the original work of Mr. Masood Ahmad under my supervision and is suitable for submission for the award of Ph.D. degree in Biochemistry.

(B.S. Srivastava)

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Masood Ahmad

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CONTENTS

PREFACE

Escherichia coli and several other microorganisms possess enzymatic system to repair damages caused by radiations, carcinogens and mutagens (Howard-Flanders, 1968; Ames, 1974; Witkin, 1976). Several genes are involved in the repair of irradiated E.coli and the same set of genes are involved in the repair of lesions inflicted by carcinogens and mutagens (Clark and Ganesan, 1975; Ishi and Kondo, 1975; Auerbach, 1976). These genes have been mapped and the products of some have been identified (Hanawalt and Setlow, 1975; Gudas and Pardee, 1975). Four repair systems have been identified: (1) Photoreactivation, (2) Excision repair, (3) Constitutive recombination repair, and (4) Inducible error-prone repair.

The primitive earth was under constant exposure of hazardous high doses of ionizing and ultraviolet radiations. While the former has high penetrating power and ionizing capabilities, ultraviolet light is selectively absorbed by DNA, the genetic material of most living organisms. Various DNA repair systems are believed to be evolved to cope with offensive exposures of ionizing and non-ionizing radiations. Because of the formation of ozone layer in the upper atmosphere, the ionizing radiations are not found now in natural environment at a value high enough to constitute a hazard to living organisms. It is, therefore, logical to question the role of

these repair systems, since an unwanted system ought to have been discarded during the course of evolution. Several speculations were made around 1970 that the DNA repair systems may be involved in normal metabolism e.g. genetic recombination (Howard-Flanders and Theriot, 1966), to cope with nonphysiological environmental conditions like pH, temperature and ionic strength (Bridges et al., 1969), and of course to alliviate the hazards of environmental mutagens (Ames, 1974; Ishi and Kondo, 1975).

We became interested in the role of DNA repair system under non-physiological conditions. Encouraged by the preliminary report of Bridges et al. (1969) on the correlation between heat and radiation sensitivities, we selected E.coli K-12 and its phage λ, and the effect of non-physiological temperature (52°C) was studied.

In the first chapter of this dissertation, damages induced by radiations and temperature, and DNA repair systems have been described.

The second chapter describes the bacterial and phage strains, composition of media and buffer.

The third chapter describes a new methodology developed for the purification of bacteriophage λ.

Fourth chapter deals with the survival of various radiation sensitive mutants of E.coli K-12 exposed to 52°C,

recovery of heated cells, effects of the metabolic inhibitors on recovery, and heat induced mutagenesis.

Fifth chapter is devoted to the description of biochemical aspects of heat-damage and repair in E.coli. This chapter describes the nature of heat-lesions in DNA, RNA, protein and cell membrane.

Sixth chapter deals with the effect of 52°C on λ.

Seventh chapter is comprised of the general discussion. The purpose of the general discussion is to coordinate very briefly the entire data and propose a model on heat lesions and their repair.

This is followed by abstract and bibliography.

CHAPTER 1

GENERAL INTRODUCTION

DNA is the genetic material of most of the living organisms. The structural integrity of DNA is therefore very essential for replication and transcription. Any kind of damage induced in the DNA by physical or chemical agents has to be removed and the structure of DNA restored. Therefore, repair of DNA is an essential function for the survival and multiplication of a living cell.

During the past several years, the chemical nature of the UV (ultraviolet radiation)-induced lesions have been precisely identified. The lesions induced by ionizing radiations are comparatively poorly understood at least as far as the base damages are concerned. It has also become known that cells possess enzymatic machinary which removes offensive lesions induced by radiations and some chemical agents (Howard-Flanders, 1968; Auerbach, 1976). The enzymatic system(s) involved in the repair of DNA are called DNA repair systems.

Molecular Biology of Radiation Repair Systems:

The earliest suggestion on recovery of bacteria after exposure to ultraviolet light was made by Hollaender and Curtis in 1935. Later, Kelner (1949) observed that exposure of UV-irradiated bacteria to visible light resulted in significant recovery and suggested that UV-lesions in bacteria can be photoreactivated. The isolation by Hill (1958) of an *Escherichia coli* mutant (B_{s-1}) which was more UV-sensitive than the parent strain, provided the first evidence on genetic control of radiation sensitivity. This strain was also

sensitive to the action of X-rays and ^{32}P suicide (Hill and Simson, 1961). Setlow and Carrier (1964) and Boyce and Howard-Flanders (1964) independently demonstrated that UV-induced thymine dimers (Fig.1) in bacterial DNA were not excised in a UV-sensitive strain but were excised in the wild-type strain. This suggested that excision of thymine dimers from bacterial DNA may be important for cell survival and that it is genetically controlled. The foregoing developments paved the way for a systematic study of DNA repair mechanisms in prokaryotes and eukaryotes, and led to the discovery of additional repair systems.

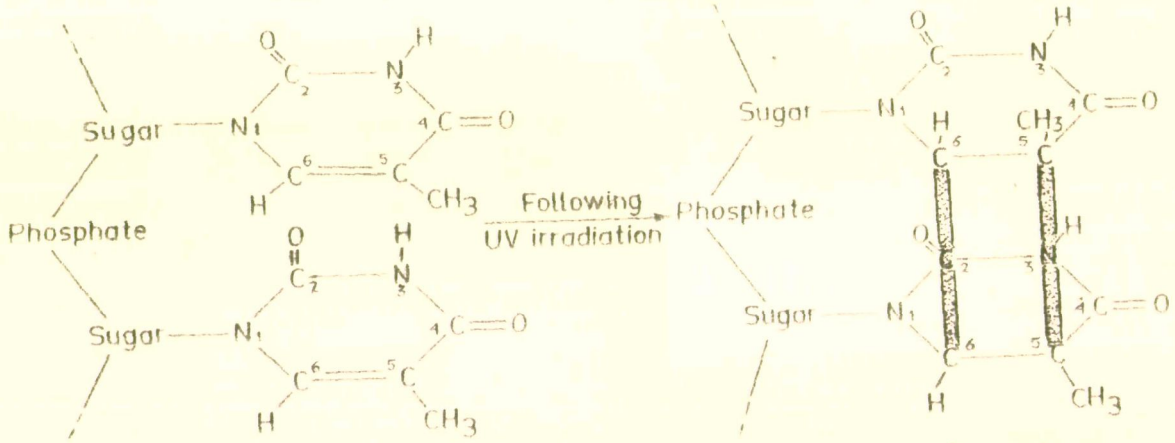
The following repair systems have been elucidated in bacteria (Fig.2):

1. Photoreactivation: This is the phenomenon of recovery in which the viability of UV-irradiated cells is restored if irradiated cells are immediately exposed to visible light.

Following the observation of Kelner (1949) and Dulbecco (1949), photoreactivation was observed in many organisms including some plants as well as animals (Goodgal et al., 1957; Cook, 1970; Rupert, 1975). Largely because of its simplicity and absolute requirement for light, it was also the first system to be observed in vitro (Rupert et al., 1958) and was the first to be characterized with regard to mechanism (Rupert 1962a,b).

Kelner (1953) showed that in E.coli, DNA synthesis is inhibited following exposure to low doses of UV radiation.

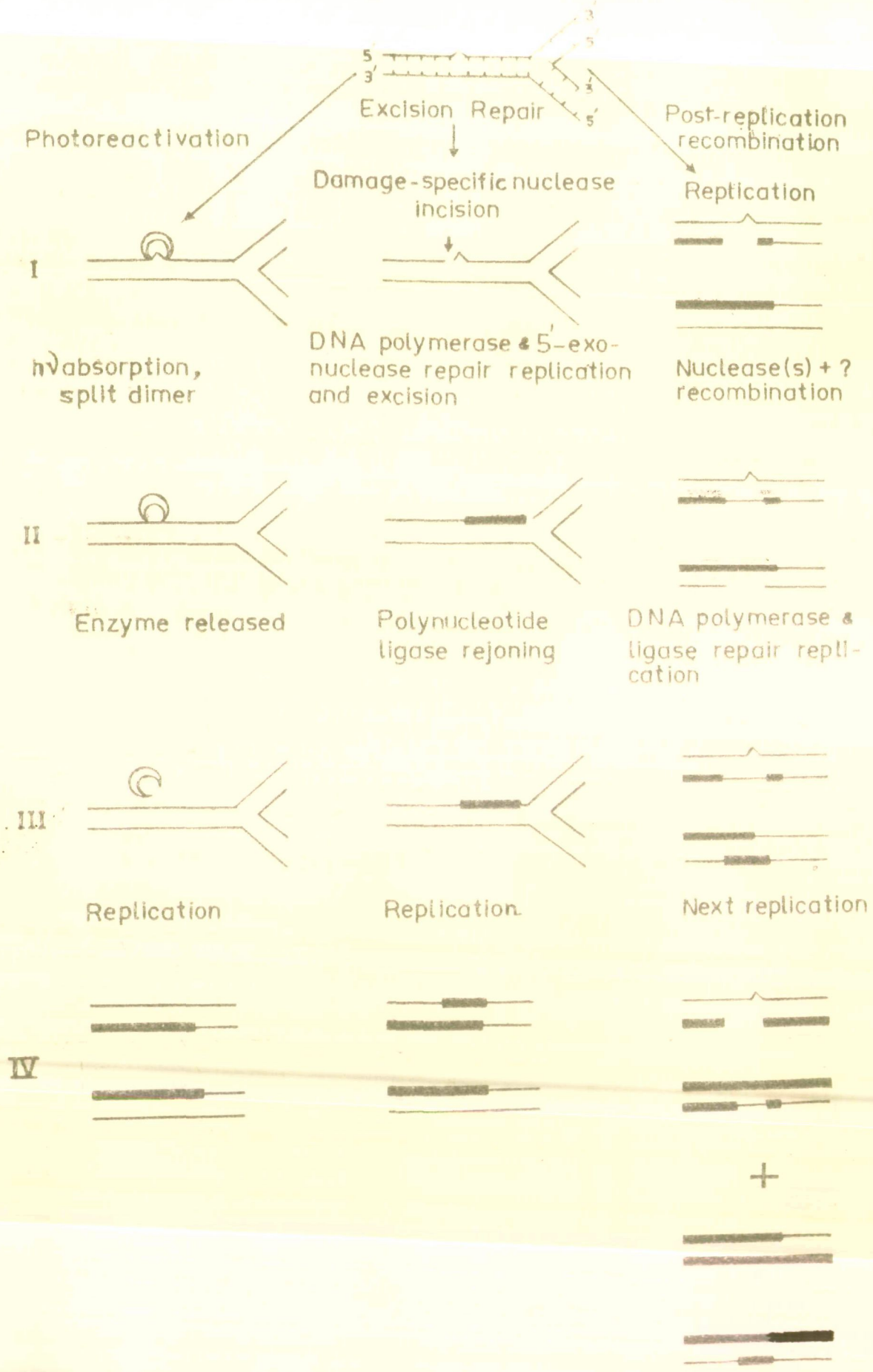
Fig. 1. Cyclobutane-type thymine dimer induced by UV light.



A portion of
DNA strand containing
adjacent thymine base

5,6 cyclobutane type
thymine-thymine dimer

Fig. 2. Scheme of the principal repair mechanisms in bacteria proposed to cope with UV-induced pyrimidine dimers in DNA. Cyclobutane dimers are represented by the inverted 'V'. Heavy lines indicate new DNA synthesis [Adopted from the paper: Hanawalt, P.C. (1975) Genetics (Suppl.) 79: 179].


5
3
Photoreactivation
Excision Repair
Post-replication recombination
Damage-specific nuclease incision
Replication
I
hν absorption, split dimer
DNA polymerase & 5'-exo-nuclease repair replication and excision
Nuclease(s) + ? recombination
II
Enzyme released
Polynucleotide ligase rejoning
DNA polymerase & ligase repair replication
III
Replication
Replication
Next replication
IV
+

This inhibition could be reversed by photoreactivation. Working with extract of yeast cells, Rupert (1960, 1961 & 1962a,b) showed that photoreactivating activity resides in an enzyme (EC 4.1.99.3) which forms a complex with UV-irradiated DNA in the dark. The complex is dissociated upon illumination to visible light (310 to 400 nm) by absorbing one quantum of light. Pyrimidine dimers have been shown to be the substrate for the PR(photoreactivating) enzyme. The PR enzyme monomerises the dimers formed by adjacent pyrimidines situated on the same strand of irradiated DNA (Wulff and Rupert, 1962; Setlow et al., 1963; Setlow, 1966). The gene controlling photoreactivation in E.coli has been mapped and designated as phr (Van De Putte et al., 1965).

Photoreactivating enzymes are widely prevalent in eukaryotes and photoreactivation has also been demonstrated in human leukocytes and fibroblasts (Sutherland, 1974). Decreased level of PR enzymatic activity was shown in Xeroderma pigmentosum cells (Sutherland and Oliver, 1975; Sutherland et al., 1975). Haemophilus influenzae lack photoreactivation, however, photoreactivation of UV-irradiated H.influenzae can be demonstrated with an extract of E.coliB (Goodgal et al., 1957). The simplicity of enzymatic mechanism and its widespread occurrence may be consistent with its early evolution as a protective system against radiation.

2. Excision repair: Ultraviolet light induced damage in cellular DNA is also repaired in dark by excision repair system (Setlow and Carrier, 1964; Boyce and Howard-Flanders, 1964).

Excision repair is the process in which the lesions in DNA are removed and the gap is refilled with correct base sequences using the opposite intact strand as template. Kondo et al. (1970) reported that excision repair enzymes could also act upon the DNA damaged by chemical mutagens and carcinogens. This repair system could have at least four steps viz. incision, excision, gap filling and DNA sealing. The plausible steps are as follows:

A specific endonuclease, known as correndonuclease, makes a nick next to the DNA lesion at 5'-site. Correndonuclease II acts on the difunctional lesions such as pyrimidine dimers induced by UV light (Braun and Grossman, 1974) whereas correndonuclease I acts on the monofunctional lesions e.g. the 2, 4-dihydroxy dihydrothymine derivatives arising from γ-irradiation. Correndonuclease II is encoded by the uvrA and uvrB genes (Braun and Grossman, 1974).

An exonucleolytic excision releases the oligonucleotide bearing the DNA lesion plus some bases on either side of it. The excision step in E.coli is believed to be performed by the 5'→3' exonucleolytic activity of DNA polymerase I (Meyneker and Klenow, 1975; Glickman, 1975). However the role of other correxonucleases can not be ruled out for the efficient repair. The uvrC gene product is an exonuclease which chews the DNA containing pyrimidine dimer. Thus it minimizes the probability of premature sealing by the DNA ligase of nicked DNA containing dimer. (Seeberg and Rupp, 1975). uvrE and mfd genes are also suggested for efficient excision repair (Siegel, 1973; Van

Sluis et al., 1974; George and Witkin, 1974, 1975).

The excision gap is patched by repair replication (Pettijohn and Hanawalt, 1964). Cooper and Hanawalt (1972a,b) have demonstrated that the size of repaired regions in *E.coli* DNA varies from short stretches of nucleotides to the stretches containing several thousand nucleotides. The final step is the sealing of the sugar-phosphate linkage by polynucleotide ligase (Sgaramella, et al., 1970).

Alternate pathways of excision repair, utilizing DNA polymerase III or DNA polymerase II have been demonstrated in mutants lacking DNA polymerase I activity. (Youngs and Smith, 1973a; Masker, et al., 1973). In addition to the short patch type of excision repair as described above, a long patch pathway has been identified which requires the product of the *recA*$^+$ and *lexA*$^+$ genes and occurs only in growth supporting media (Cooper and Hanawalt, 1972a; Youngs and Smith, 1973b; Youngs et al., 1974). It has also been shown that excision repair requires ATP (Seeberg and Strike, 1976).

Specific endonucleases have recently been isolated and purified from bacteria which act on chemically or physically distorted DNA (Carrier and Setlow, 1970; Nakayama et al., 1971; Radman, 1976). T4 encoded exonucleases may excise both the host and viral DNA whereas *E.coli* nuclease could not excise lesions from T4 DNA (Setlow, 1966; Onahima and Sekiguchi, 1972).

Excision repair has been shown to be defective in certain

strains of E.coli K-12 which are designated as uvr mutants. These mutations have been mapped at 5 loci, namely uvrA, B, C, D and E. The classical mutant strain E.coli B_{s-1}, isolated by Hill (1959) is also defective in excision repair. Mutation in any of the three loci uvrA, B and C makes the strain deficient in excision of pyrimidine dimers (Howard-Flanders et al., 1966). Strains with uvrD mutation are somewhat less sensitive to UV but are more sensitive to X-rays (Ogawa et al., 1968). The $uvrE^+$ allele is also required in excision repair and most probably controls in some way repair replication (Sinzinis et al., 1973; Smirnov et al., 1973a). The uvrE mutation also leads to the mutator phenotype, increasing the probability of A.T $\rightarrow$ G.C $\rightarrow$ A.C substitutions (Smirnov et al., 1973b).

3. Post-replication recombinational repair: The DNA lesions, especially the UV-induced pyrimidine dimers that are neither photoenzymatically split nor removed from the DNA by excision repair (as in excision-deficient uvr mutants kept in the dark), block the continuous progress of the DNA replication fork, but do not prevent reinitiation of DNA synthesis at a point beyond the dimer (Rupp and Howard-Flanders, 1968). When lesions are produced in DNA by UV, daughter strands are detected initially as segments of relatively low molecular weight. Their continuity is interrupted by gaps of about 1,000 nucleotides long (Iyer and Rupp, 1971) and are situated opposite the lesions in parental strand (Howard-Flanders, et al., 1968; Sonbeu, et al., 1974). The daughter strand gaps are secondary lesions caused by the replication of DNA containing primary photoproducts

that prevent base pairing. The third type of enzymatic DNA repair, by which the molecular weight of the newly synthesized strand increases, is called post-replication repair. This type of repair was first demonstrated in E.coli by Rupp and Howard-Flanders (1968). It operates to connect the daughter-strand segments, thereby endowing them with the continuity required for further replication. Although first demonstrated in uvr⁻ strains, post-replication repair occurs also in uvr⁺ strains capable of excising pyrimidine dimers efficiently (Smith and Meun, 1970; Sedgwick and Bridges, 1974). It implies that ability to perform excision repair does not prevent some non-instructive lesions from passing through a replication fork. In excision deficient (uvr) mutants that are otherwise normal, the major mechanism of post-replication repair is recombinational, requiring the activity of rec⁺ gene. A hint of possible involvement of recombination in the process of repair after ultraviolet irradiation was first obtained when multiplicity reactivation was discovered (Luria, 1947). After UV-irradiation followed by DNA replication and post-replication repair, specifically labelled parental DNA is found covalently inserted into the daughter strands. The number of insertions corresponds approximately to the number of daughter strand gaps initially produced (Rupp et al., 1971).

The post-replication repair occuring in mammalian cells is a little different from that occuring in E.coli. In mouse, Lehman (1972) observed that during post-replication repair, gaps opposite pyrimidine dimers are left as in E.coli, but are

filled subsequently not by transfer of intact single-strand segments, but by de novo synthesis. However, Painter (1974) suggested that the gaps as observed in irradiated bacteria do not occur in mammalian system.

Two models have been proposed to explain post-replication repair, "gapped synthesis" (Rupp and Howard-Flanders, 1968) and "replicative by pass" (Higgins et al., 1976; Fujiwara and Tatsumi, 1976). These two models would suggest that if DNA was replicated, daughter strands would be synthesized in short pieces according to gapped synthesis or would be intact according to replicative by pass. However, if post-replication repair was blocked, fork progression would be blocked in replicative by pass but not in gapped synthesis.

E.coli is capable of performing post-replication repair via a number of distinct pathways. RecA bacteria are deficient in post-replication repair, however mutation in $recF^+$, $recC^+$, $lexA^+$, $recF^+$ and $polA^+$ all genes are also known to affect post-replication repair (Tait et al., 1974; Sedgwick and Bridges, 1974; Sedgwick, 1975a,b; Rothman et al., 1975; Youngs and Smith, 1976). In $recA^-$ mutant no post-replication joining of daughter strands occur (Smith and Meun, 1970). The requirement for the $recA^+$ gene product, which is necessary for any type of genetic recombination in E.coli (Clark, 1973) does not necessarily imply that all pathways of post replication repair are recombinational (Witkin, 1976). All the replication repair pathways so far discussed, except the one requiring both $recA^+$ and lex^+ gene activities, are probably constitutive and error free repair systems.

The recombination deficient, the so called rec mutants (rec-A,B,C), were first isolated by Clark and Marguilies (1965). Lloyd and Low (1976) proposed that the recA gene product is involved in the initiation of cross over between homologous DNA molecules. RecF mutant was isolated by Horii and Clark (1974). Mutation in rec genes causes increased sensitivity to UV and X-rays (Howard-Flanders and Theriot, 1966). Rec$^-$ strains are sensitive to the lethal action of radiations and are refractory to the mutagenic action of radiations and to several other chemical agents (Miura and Tomizawa, 1968; Witkin, 1969). The lex (exr mutants of E.coli B) mutants are sensitive to X-rays and are nearly recombination proficient. Mutations lon (or filB) make the strain sensitive to UV and X-rays. PolA (DNA Polymers I deficient) strain is UV sensitive but polB (polymerase II deficient) strains are not (Campbell et al., 1972). The polymerase III (dna II mutation) may be a back up enzyme (Tait et al., 1974; Hamelin et al., 1976). It is also required for growth medium dependent recovery (type III) and is necessary for growth medium independent repair (type II) in polA cells. (Hamelin et al., 1976).

4. Inducible error-prone repair: the 'SOS' repair hypothesis: An error-prone repair hypothesis, which is called as 'SOS' repair has been proposed by Radman (1974, 1975) and was experimentally supported by Witkin (1976). Main features of the 'SOS' repair are, (1) it is induced or activated following damage to DNA, (2) it requires de novo protein synthesis, (3) it requires several genetic functions, among them the best studied are

recA$^+$ and *lex*$^+$ of *E.coli*, and (4) the physiological and genetic requirements for the expression of 'SOS' repair are suspiciously similar to those necessary for the prophage induction.

Defais *et al.* (1971) proposed that UV mutagenesis in *E.coli* might depend upon an inducible function which, like prophage, is repressed in healthy wild-type cells but is expressed in response to UV-irradiation. One basis for this suggestion was the dependence of UV-mutagenesis upon *recA*$^+$ and *lexA*$^+$ gene products, which are also required for prophage induction by UV-radiation, and for the other diverse functions such as inhibition of septum formation, inhibition of respiration, the increased production of *recA* gene product and the Weigle's reactivation (Witkin, 1976). Taken together, these processes are called as 'SOS' response (Radman 1974, 1975; Witkin, 1976). 'SOS' functions are not expressed in cells carrying either *recA* or *lexA* mutation (Witkin, 1976). The hypothesis that the *recA*$^+$ and *lexA*$^+$ gene products jointly control a coordinately regulated group of inducible functions including an error-prone DNA repair activity is known as 'SOS' hypothesis. The designation 'SOS' (the international distress signal) implies that damage to DNA or stalled DNA replication initiates a regulatory signal (also known as 'SOS' signal) that causes the simultaneous derepression of various functions, all of which presumably promote the survival of the cell or its phages.

The idea of 'SOS' hypothesis is the consequence of many early observations. Lwoff *et al.* (1950) observed that UV radiation initiates mass induction of prophage in lysogenic

bacteria. Other treatments, with ability to stop DNA replication, were also found to cause lysogenic induction, for example, exposure to X-rays (Lataraet, 1951), incubation with mitomycin C (Otsuji et al., 1959), starvation for thymine (Malechen and Skaar, 1962; Siegrd and Deveret, 1962), temperature elevation in certain mutants unable to synthesize DNA at high temperature (Monk and Gross, 1971; Krcek and Claus, 1972; Schuster et al., 1973). Weigle (1953) observed substantial enhancement of plaque forming ability of UV-irradiated bacteriophage λ when the host bacterium was also UV-irradiated prior to infection. This increase in survival as compared to infection with unirradiated host was also accompanied with high frequency of mutation. This finding provided the basis for inducible error-prone repair now commonly known as 'SOS' repair. This reactivation, now termed as Weigle's reactivation or W-reactivation, requires *recA*$^+$ (Miura and Tomizawa, 1968) and *lexA*$^+$ (Defais et al., 1971) genotypes of the host.

Mutagenic W-reactivation of bacteriophage λ is independent of both excision repair and recombinational repair and that it depends upon a novel error-prone repair activity induced or activated in the host cell by UV-radiation and other SOS-inducing treatments (Deveret et al., 1975). The requirement for new protein synthesis after the inducing treatment suggests induction rather than activation of this error-prone repair (Ono and Shimazu, 1966; Bridges, 1966). Indeed a protein designated as 'X' has been observed to be induced following SOS inducing treatments to wild-type bacteria but not in *recA*$^-$ or

lex⁻ bacteria (Inouye and Pardee, 1970; Inouye, 1971; Tudas and Pardee, 1975; West and Emerson, 1977). Protein X is responsible for the proteolytic cleavage of λ -phage repressor in induced lysogens (Mount *et al*., 1975; McIntee *et al*., 1976; Emerson and West, 1977). Fauquet *et al*.(1977) observed that DNA polymerizing activity was induced in UV-irradiated *E.coli*.

There is considerable evidence that spontaneous mutation rates are greatly affected by changes in the relative efficiency of polymerizing and proof reading activities in DNA polymerases (Drake, 1970; Kornberg, 1974). Villani *et al*.(1978) have suggested that treatment of DNA damaging agents induce an inhibitor which inhibits or reduces the proof reading 3'→5' exonucleolytic activity of constitutive DNA polymerases.

A radiation sensitive strain mutated in gene *rar* of *E.coli* K-12 was described by Srivastava (1976) whose radiation sensitivity was associated with DNA replication. It was proposed that *rar*⁺ gene controls the replication of damaged DNA and thus regulates the coordination between repair and replication of damaged DNA. It has been suggested that *rar*⁺ is an inducible gene which is derepressed as the 'SOS' signal is released due to imbalance in repair and replication (Srivastava, 1978). The *rar*⁺ gene product does not participate in repair directly but directs a control system on replication of damaged DNA.

Repair of bacteriophage λ :

The damages induced in λ DNA by ultraviolet radiation are repaired utilizing one or more of the three processes:

(1) host cell reactivation (Jaren and Zinder, 1955; Harm, 1963),
(2) prophage reactivation (Jacob and Wollman, 1953), and
(3) Weigle reactivation (Weigle, 1953).

Host cell reactivation is the capacity of unirradiated host to reactivate irradiated phage. Bacteria proficient in host cell reactivation are referred to as HCR^+. Excision defective strains are HCR^-. This is the most efficient repair process which is error-free and non-mutagenic (Harm, 1963). Bacterial genes involved in repair of bacterial DNA also effect repair of λ DNA. Host cell reactivation of phage has been demonstrated to be due to excision of pyrimidine dimers (Boyle and Setlow, 1970).

Prophage reactivation is the process of DNA repair of UV-irradiated λ when the host bacterium contains the hetero-immune homologous phage. This type of reactivation does not occur if the host is non-lysogenic or lysogenic with non-homologous phage (Jacob and Wollman, 1953). This phenomenon is believed to be due to recombination between the homologous DNAs or UV-damaged phage and the intact resident phage (Chase, 1964; Devoret and coquerelle, 1966; George and Devoret, 1971). This process makes use of recombination repair enzymes of host (Howard-Flanders and Theriot, 1966) and *red* gene of phage λ but not the *int* gene (Blanco and Devoret, 1973). No UV-mutagenesis seems to result by prophage reactivation (Miura and Tomizawa, 1970; Blanco and Devoret, 1973). Multiplicity reactivation (Luria, 1947) and cross-reactivation or marker rescue (Doermann,

1961) have also been suggested to be due to recombination. There is in principle no need of extra copies of DNA strands. Thus, these processes are believed to be presynlicative recombination repair (Baker et al., 1971; Howard-Flanders and Lin, 1973; Devoret et al., 1975).

Weigle-reactivation is defined as the increased survival of UV-irradiated phage λ when the host bacterium is also exposed to UV prior to infection (Weigle, 1953). This phenomenon is accompanied by high frequency of mutation.

Devoret et al.(1975) have suggested that W-reactivation of bacteriophage λ is independent of both excision and recombinational repairs and that it depends on a new error-prone repair activity induced in the host cell by UV-radiation and other 'SOS-inducing' treatments. Error prone SOS-repair hypothesis (described above) is based on this very finding of W-reactivation of phage λ. W-reactivation does not occur if the chloramphenicol is present in the pre-infection incubation medium (Defais et al., 1976). W-reactivation of UV-irradiated λ does not require the function of uvr and pol genes but it requires functional rec and lex genes.

Bacteriophage λ which makes use of bacterial machinary for its transcription and maturation, also codes at least in part its own repair system which appears to complement with that of bacteria. Two genes of phage λ have been shown to influence radiation sensitivity. These are designated as red and gam. The red and gam play a more significant role in replication

birth and maturation of the phage during normal vegetative growth.

The red⁻ strain of λ makes the phage totally recombination deficient in recA host, in the wild-type bacteria vegetative recombination is also affected (Echols and Gingery, 1968; Signer and Weil, 1968). The red gene codes for two proteins, exonuclease V and β-protein. The former is considered equivalent to recBC coded DNase. The deficiency in gene red renders phage sensitive to γ-rays (Srivastava, 1973). It has been shown in alkaline sucrose gradients that γ-rays induced ssb (single strand breaks) may be repaired by red⁺ pathway (Srivastava, 1973). Red-dependent radioresistance could be restored if exonuclease V and β-protein were present during recovery even after 1 hr of irradiation (Trgovcevic and Rupp, 1975).

The gam mutant was isolated by Zissler et al. (1970) and was shown to cause a slight recombination deficiency in phage λ . It was later shown that γ-protein interacts with the recBC nuclease (Unger and Clark, 1972; Trgovcevic and Rupp, 1974).

The gam mutants are only marginally UV-sensitive in a uvrA recA strain. However, these mutants have been shown to be sensitive to X-rays in wild-type and DNA polymerase I deficient strains (Trgovcevic and Rupp, 1975). Both the gam and red genes, have been assumed to act independently (Trgovcevic and Rupp, 1975).

Human genetic diseases involving defective DNA repair

Six human genetic diseases i.e., Xeroderma pigmentosum (XP; Siemens and Kohn, 1928), Bloom's syndrome (BS; Bloom, 1954), Ataxia telangiectasia (AT; McFarlin et al., 1972), Fanconi's anaemia (FA; Fanconi, 1927), progeria, or Progeroid syndrome (PS; Cleaver, 1968), and Retinoblastoma (Weichselbaum et al., 1978) appear to be defective in DNA repair.

XP patients are characterized by defective pigmentation of the body. The XP cells are sensitive to sunlight and mutagenic agents and the patients suffer from all types of skin cancer causing early death (Robbins et al., 1974; Lo and Stitch, 1975). The XP condition is inherited as an autosomal recessive gene and is associated with various deficiencies in DNA repair capacity (Cleaver, 1968, 1969; Cleaver and Footema, 1975). In one type of XP, ability to perform the first step of excision repair (i.e. incision) is absent (Setlow et al., 1969) as in uvr mutants of E.coli. Tanaka et al. (1975) have reported that UV-induced unscheduled DNA synthesis in all complementation groups of XP cells could be restored by simultaneous treatments with T4 endonuclease V and UV-irradiated Sendai virus. These results suggest that XP cells are deficient in the function equivalent to endonuclease V. On the contrary, the other individuals have been found to be normal in excision of UV-induced pyrimidine dimers and defective in post-replication repair (Lehman et al., 1975; Fornace et al., 1976). Skin fibroblasts taken from a XP

patient having defective incision of pyrimidine dimers, were also found to be UV hypermutable at low doses of radiations (Maher et al., 1976). Hypermutability of XP cells may contribute to high rate of carcinogenesis. Setlow et al. (1976) suggested that XP cells are defective in repairing a component of anoxic γ-ray damage. Repair of DNA damage by alkylating carcinogens was also found to be defective in XP derived fibroblasts (Goldstein, 1977).

Bloom's syndrome and Fanconi's anaemia are associated with high frequencies of chromosomal aberrations (Howard-Flanders, 1973). Hand and Gorman (1975) observed that Bloom's syndrome patients could have low level of replication of their dermal fibroblasts. The low level of DNA replication could be due to delayed DNA-chain maturation. Moreover, these cells were also found to be UV-sensitive (Gianelli, et al., 1977). Cells from patients suffering from Fanconi's anaemia were normal in this respect (Hand and Gorman, 1975).

Fanconi's anaemia is autosomal recessive disease of man characterized by a progressive hypoplastic pancytopenia associated with diverse congenital anomalies, spontaneous chromosome breakage and predisposition to leukaemia and other cancers. Lymphocytes from FA patients were found to be excessively susceptible to chromosome breakage by di- or polyfunctional alkylating agents, and this was interpreted as a possible indication of defective DNA repair of the FA cells (Sasaki and Tonomura, 1973). Latt et al. (1975) have reported

lower sister chromatid exchange response in FA lymphocytes following mitomycin C treatment, although there is marked increase in chromatid breaks and rearrangements. FA fibroblasts were found to be susceptible to chromosome damage by carcinogens (Auerbach and Wolman, 1976). Sasaki (1975) suggested that FA cells are defective in the repair of DNA interstrand cross-links.

Ataxia telangeictasia (AT) is also an autosomal recessive defect showing the following clinical features: Cerebellar ataxia, telangeictasia, IgA deficiency, an enhanced frequency of malignancy and an enhanced level of spontaneous chromosome instability (Mafarlin et al., 1972; Harnden, 1974). All cell lines are more susceptible to X-rays (Cunliffe et al., 1975; Taylor et al., 1975) and exhibited increased chromosome aberrations induced by ionizing radiations in leukocyte culture from AT patients (Hatcher et al., 1976).

Progeria is characterized by premature ageing and early death (DeBusk, 1972), and assumed to be associated with autosomal recessive factors (Viegas et al., 1974). It has been reported to be defective in repair of X-rays (Epstein et al., 1973) but contrary report by Regan and Setlow (1974) showed no increase in X-ray sensitivity.

Recently, Weichselbaum et al. (1978) have reported that the patients with hereditary retinoblastoma were more sensitive to X-rays than those having sporadic blastoma. Moreover, defective DNA repair was postulated to account for the

high incidence of second tumor in these patients.

These DNA repair diseases are very rare. These are also autosomal recessive conditions that only produce symptoms in individuals who receive two copies of the defective gene. Persons with only one copy are rare gene carriers passing the gene to their progeny but who do not themselves develop symptoms. Even though the homozygotes are rare, the carrier may be relatively common in population, which probably might be the cause of high incidence of cancer (Marx, 1978).

DNA lesions:

One of the paradoxes in the field of DNA repair is that more is known about repair pathways than about the lesions on which they operate. Most physical and chemical agents reacting with DNA, induce a large variety of products. In many cases lesions induced by different agents are structurally related and are expected to have similar effects on the local conformation of DNA helix (Painter, 1978). Structurally related lesions are also expected to have similar biological effects regardless of the agents responsible for their formation (Cerutti, 1978). Considerably more is known about the lesions introduced by ultraviolet light and certain alkylating agents than those produced by ionizing radiation or heat-treatment.

As far as the structural classification of DNA base damages is concerned, it can be categorized under the following headings:

1. Monofunctional lesions causing negligible helix distortion or functionally tolerable damage (Cerutti, 1974).

Products with unaltered base pairing and base-stacking capacities (e.g. 7-alkyl guanine formed by most alkylating agents; 5-hydroxymethyl or, 5-hydroperoxymethyl thymine formed by ionizing radiations).

2. Monofunctional lesions causing minor helix distortion or 'ionizing type' (Regan and Setlow, 1973).

A. Products with only slightly altered base-pairing and base stacking properties; ring saturation products (e.g. pyrimidine photoproducts of the 5, 6 dihydroxy-dihydrothymine type induced by ionizing radiations).

B. Ring contraction, ring fragmentation and ring-elimination products (e.g. ring fragmentation products of pyrimidines and purines produced by ionizing radiations; ring elimination leading to apurinic and apyrimidinic sites induced by ionizing radiations; ring elimination leading to apurinic and apyrimidinic sites induced by alkylating agents, ionizing radiations or heat).

3. Lesions causing major helix distortion or 'ultraviolet type' (Regan and Setlow, 1973).

A. Monofunctional lesions with bulky substituents or substantially altered base-pairing properties; intercalation damage etc.

B. Difunctional lesions (e.g. inter-strand and intra-strand cross-linking of neighbouring bases; pyrimidine dimers; cross-linking between DNA and proteins).

Monofunctional lesions are the damages involving only single base while difunctional lisons are those which affect two bases simultaneously forming covalent bond between them. Detailed review on this topic has been given by Cerutti (1975). Lesions induced by UV, ionizing radiations and heat are summarized as under:

(a) <u>UV-induced lesions</u>: UV-light is selectively absorbed by DNA in living cells and 98% of the UV-induced lesions constitute cyclobutane type pyrimidine dimers (Fig. 1). The pyrimidine dimers formed in the DNA of irradiated bacteria appear to comprise 50% thymine-thymine, 40% thymine-cytosine and 10% cytosine-cytosine dimers. The dimers have been extensively studied both <u>in vitro</u> as well as <u>in vivo</u> (Setlow and Setlow, 1972; Cleaver, 1974; Patrick and Rahn, 1975). Less predominent and rarely reported UV-lesions, mostly demonstrated <u>in vitro</u> conditions, comprised of thymine dimers of other than 5,6 unsaturated linkage of cyclobutane type (Donnellan and Setlow, 1975; Varghese and Patrick, 1969; Varghese, 1970), unzipping of DNA strands in the vicinity of pyrimidine dimers (Gupta and Mitra, 1974), interstrand cross-linking (Glisin and Doty, 1967), and cross-links between DNA and proteins in the case of bacteria-phages (Smith, 1962; Smith and O'leary, 1967). But these lesions are insignificant at biological doses of UV.

(b) Ionizing-radiation induced lesions: Ionizing radiations induce a number of damages in DNA. Single strand breaks, alkali labile sites, double strand breaks (Collyns et al., 1965; Ougle, et al., 1976), and base damages of numerous yet unidentified types (Friefelder, 1965, 1966; Ginoza, 1967; Setlow and Setlow, 1972; Cerutti, 1974). The single strand breaks are considered to be repairable lesions (Town et al., 1971), whereas the double strand breaks and base damages are considered to cause lethality. Glycosidic bond cleavage has been observed in double stranded DNA at neutral pH (Ljungquist et al., 1974). Apurinic and/or apyrimidinic sites are present immediately after γ-irradiation, and incubation of the irradiated DNA in neutral solution resulted in increase of such sites (Lindahl and Ljungquist, 1975). Ionizing radiations have also been reported to alter the membrane of irradiated bacteria (Alper, 1968; Kada et al., 1970; Kada, 1970; Crump et al., 1972; Nair et al., 1975; Nair and Pradhan, 1976; Mitton, 1976).

Some authors have suggested increased radiation sensitivity correlated with increased strand breaks in oxic compared to anoxic condition (Town et al., 1972; Johansen et al., 1974; Johansen and Saye, 1973). Lack of Oxygen effect was reported earlier for bacteriophages ES and T7 (Freifelder, 1966; Neary et al., 1970). Dean et al. (1969) were the first to suggest that oxic and anoxic irradiation yielded identical number of breaks, and that anoxic breaks were subject to rapid

enzymatic repair. Srivastava (1974) reported that there was no difference in the number of anoxic and oxic breaks in phage λ DNA irradiated extracellularly. He found that DNA polymerase I repairs strand breaks very fast during irradiation and before lysis in buffer and most of the anoxic breaks were rejoined by DNA polymerase I. Sapora et al. (1975, 1976), and Fox et al. (1976) using rapid lysis technique confirmed the results of Srivastava, and further suggested that the basic difference between oxic and anoxic breaks lies in the chemical nature of the breaks and events.

(c) Heat-lesions: Heat-damage involves the multitarget destruction of living cells. It affects the viability of cells due to the damages in DNA, RNA, proteins and cellular membranes (Strange, 1976; Busta, 1976; Hurst, 1977).

Effect of super-optimal temperatures on DNA revealed that there occurs spontaneous hydrolysis of DNA between 80 - 100°C (Eigner et al., 1961), depurination (Greer and Zamenhof, 1962), thermal denaturation (Ginoza and Zimm, 1961; Palecek, 1965; 1968; Das Gupta et al., 1969; Vorlickova and Palecek, 1970), in vivo thermal degradation of DNA (Sedgwick and Bridges, 1972), and induction of single and double strand breaks at 52°C (Bridges et al., 1969b; Woodcock and Grigg, 1972). However, the strand scissions depend on the strain and physiological state of the organism (Sedgwick and Bridges, 1972). No such breaks were found in vitro. At 52°C bacteriophage DNA exhibits regions of transient local denaturation at

characteristic points along the chromosome (Inman, 1966). It is believed that strand breakage is enzymatic (Goldmark and Linn, 1970; Sedgwick and Bridges, 1972). Lindahl and Ljungquist (1975) suggested that heat-induced strand-breaks in neutral solution is also primarily a consequence of depurination.

The indirect effect of heat on cellular DNA includes interruptions of nuclear DNA synthesis in protozoa and mammalian cells (Evenson and Prescott, 1970). An influence of temperature on the frequency of crossing over and mutation in heated bacteria (Zamenhof and Greer, 1958, Zamenhof, 1960), yeast (Sherman, 1968; Frascino and Moustacchi, 1972), and bacteriophages (Freese, 1961; Drake and McGuire, 1967; Baltz et al., 1976; Bingham et al., (1976) have been reported. Wood (1956) has shown that there is a dependence of thermosensitivity upon the degree of ploidy. Frascino and Moustacchi (1972) reported variation in mild heat (52°C) sensitivity during the cell cycle of Saccharomyces cerevisiae irrespective of genetic markers. In E.coli, mutations at recA, recA, exr (Bridges et al., 1969a,b) and lig (Pauling and Beck, 1975) loci were reported to increase heat sensitivity.

Other than DNA, the most frequent heat-damage include the alteration in the permeability behavior and damage to RNA (Hurst, 1977).

Damage to cell membrane has been demonstrated by decreased salt-tolerance of the organisms (Busta and Jezeski;

1963; Stiles and Litter, 1965; Landolo and Ordal, 1965; Clark et. al., 1968; Beuchat and Lechowich, 1968; Clark and Ordal, 1969; Adam, 1973; Smolka et al., 1974). In some of the rare electron microscopic studies done with heat injured cells of Bacillus Cereus, membrane was shown to have holes and fractures as a result of heating (Silva and Sousa, 1972). Heat injured cells also become sensitive to antibiotics to which the normal cells are resistant (Tsuchido et al., 1978; Flowers and Adams, 1976; Hurst et al., 1976). Loss of 260 and 280 nm absorbing material is the most common observation with injured microbes (Landolo and Ordal, 1966; Lee and Goepfert, 1975). Loss of Na^+, K^+ and Mg^{2+} has also been reported (Landolo and Ordal, 1966; Haight and Morita, 1966; Hurst et al., 1974). The loss of lipids and phospholipids from cell membranes in Streptococcus aureus and Salmonella typhimurium suggested membrane damage (Tomlins et al., 1972; Hurst et al., 1973). Recently Grau (1978) studied the transport kinetics of heat stressed E.coli at different temperatures, using lactose, proline and alpha-methylglucoside, ~~and alpha-methylglucoside~~, and concluded that cells made nonviable by heating at 50°C still have significant membrane integrity.

Sucrose gradient centrifugation of ribosomes from heat injured S.aureus, S.typhimurium B, and E.coli showed that the 30S particles were damaged or destroyed (Tomlins and Ordal, 1971; Rosenthal et al., 1972; Miller and Ordal, 1972; Weiss and Tal, 1973). This was due to destruction of the 16S ribonucleic acid species. Loss of viability after thermal stress in Aerobacter aerogenes was correlated with ribosomal

damage (Strange and Shon, 1964). However, they suggested that primary event was loss of Mg^{2+} and ribosomal damage was the consequence of this leakage. Recently Hurst and Hughes (1978) observed that brief heating to 52°C in phosphate buffer followed by incubation at 37°C caused Mg^{2+} loss and RNA destruction, and repair did not occur in S.aureus in presence of actinomycin D. However, when piperazine or Tris-Mg buffer was used as the heating menstrum neither Mg^{2+} nor RNA was lost. Salt tolerance was not affected and repair occurred in presence of actinomycin D. Heinis et al. (1978) have reported that neither nucleic acid nor Mg^{2+} leakage happened during heat treatment of Vibrio parahaemolyticus.

Heat-treatment caused generalized protein denaturation which was correlated with cell death (Rosenberg et al., 1971). Mild-heating inactivates many enzymes, particularly dehydrogenases of S.aureus and S.typhimurium (Pierson and Ordal, 1971). Activity of most (but not all) enzymes of S.aureus was restored during recovery (Tomlins et al., 1971). Active transport of certain sugars and amino acids is greatly diminished or abolished in heated cells and apparently does not become fully functional in repaired organisms (Hurst et al., 1973).

Heat treatment causes lag in cell division (Mershey, 1939), alteration in nutritional requirement (Allgood and Russel, 1969b; Gomez and Sinskey, 1973), and increased uptake of glucose (Pierson and Ordal, 1971). ATP may also be required for heat recovery (Shibasaki and Tsuchido, 1973).

Plan of work:

Aforementioned survey of literature enabled us to study the following aspects of heat sensitivity and its recovery.

i) What are the bacterial and phage genes controlling heat sensitivity to 52°C in E.coli K-12 and bacteriophage λ .

ii) What are the heat-lesions in DNA and how are these repaired in vivo.

iii) What are the physiological conditions modifying heat sensitivity.

iv) What are the biosynthetic pathways required for post-treatment liquid-holding recovery.

v) What are the heat-targets other than DNA (e.g. RNA, protein and membrane) which influence the viability of heated E.coli K-12.

CHAPTER II

BACTERIAL AND PHAGE STRAINS, MEDIA AND BUFFERS

(a) Bacterial strains: The *E.coli* K-12 strains used in this study have been tabulated as under:

Strain Designation	Relevant Genetic Marker	Source
AB1157	*thi-1, argG, thr-1, leu-6, arg-12, his-4, lac, F^-, str^r λ^s*	P.Howard-Flanders
AB1157*lac*$^+$	*thi, argG, thr, leu, proA, his,* F^-, *str^r* λ^s	This work
AB2463	*recA13, thi, argG, thr, leu, proA, his,* F^-, *str^r* λ^s	P.Howard-Flanders
AB2470	*recB21, thi, argG, thr, leu, ProA, his,* F^-, *str^r* λ^s	P.Howard-Flanders
AB1886	*uvrA6, thi, argG, thr, leu, ProA, his,* F^-, *str^r* λ^s	P.Howard-Flanders
AB2480	*uvrA6, recA13, thi, argG, thr, leu, proA, his, lac,* F^-, *str^r* λ^s	P.Howard-Flanders
AB2494	*lexA, thi, thr, leu, proA, his, metB, lac,* F^-, *str^r* λ^s	P.Howard-Flanders
JG112	*polA1, thi, thyA,* F^-, *str^r* λ^s	J.Gross
C600	*thr, leu, thi, lac,* λ^s	R.Thomas

Abbreviations:

thi = thiamine; arg = arginine; thr = threonine; leu = leucine; pro = proline; his = histidine; lac = lactose; F^- = female; λ^s = λ sensitive; str^r = streptomycin resistant; thy = thymine; met = methionine

(b) Phage strains: Strains of phage λ used in this study are tabulated as under. All the strains were obtained from Dr. R. Thomas.

Strain designation	Description
λ *g*$^+$ or λ$^+$	Wild-type strain. It forms turbid plaques on all the *E.coli* K-12 strains described above.
λ *gl*857	Conditionally defective *gl* mutant. The strain codes for a temperature-sensitive immunity repressor. Lytic cycle at 42°C and lysogenic at 32°C.
λ *gl*857 *redl*	Recombination-defective mutant. It is defective in exonuclease V and β-protein. It is also having a temperature sensitive *gl* mutation and does not form detectable plaques on *polA1* mutant of *E.coli* K-12.
λ *bio1*	Recombination deficient mutant, i.e. *red*$^-$ *gam*$^-$. It does not multiply in *recA* mutant of *E.coli* K-12.
λ *b2*	Deletion mutant, also known as density mutant. It lacks the immunity region between *J* and *gIII* of λ chromosome and, therefore, is unable to attach with the bacterial chromosome. It gives clear plaques on *E.coli* K-12.

Strain designation	Description
λvir	Virulent strain. It contains an absolute defective mutation in the immunity region and, therefore, forms clear plaques.
λg90	It is mutated in the g region. It forms clear plaques on E.coli K-12.

(c) Maintenance and growth of bacteria: Each strain of E.coli was streaked on tryptone agar plates (TA12). A single colony was picked up and repurified by streaking on agar plate. A single colony was picked up again and the genetic marker associated with that strain was tested. Having satisfied, the culture raised from that clone was streaked on tryptone agar slants (TA12), allowed to grow overnight at 37°C and stored at 4°C. Every month cultures were transferred on fresh slants.

Overnight culture was raised in tryptone broth at 37°C. The culture was diluted 50-times in fresh broth and shaken at 37°C till cell number reached about 2×10^8 cells/ml. Such cultures were used in all the experiments.

(d) Maintenance and preparation of phage lysate: It has been described in chapter VI.

(a) Media:

(1) Minimal medium. The composition of the minimal medium abbreviated as MM in this thesis is as under:-

Required amino acids	...	...	40 µg/l (each)
$K_2HPO_4.3H_2O$	...	...	7.0 g/l
KH_2PO_4	...	...	3.0 g/l
$(NH_4)_2SO_4$	...	...	1.0 g/l
$MgSO_4.7H_2O$	...	...	0.5 g/l
Sodium citrate	...	...	0.5 g/l
$MnSO_4$	...	...	0.1 ml/l of (1 mg/ml)*
$Fe_2(SO_4)_3.H_2O$	...	...	0.1 ml/l of(1 mg/ml)*
Vitamin B_1	...	...	0.5 ml/l of (1 mg/ml)*
Glucose	...	...	100 ml/l of (10% w/v)*

* The quantities in the parentheses indicate the strength of the stock solution. All these stock solutions were strelized separately at 15 lb/sq inch for 15 min and added at the time of use.

(2) Rich medium (= Recovery medium = tryptone both = nutrient broth).

This medium is frequently abbreviated in this thesis as RM and BHB. The composition of the medium is as follows:

Tryptone	...	...	10 g/l
Yeast extract	...	...	5 g/l
Sodium chloride	...	...	5 g/l

Plating medium was prepared by adding 1.5% (w/v) agar to the above constituents.

(3) TA7: The composition of TA7 used for phage work is as under:

Tryptone	...	...	10.0 g/l
Sodium chloride	...	...	2.5 g/l
Agar-agar	...	...	7.0 g/l

(4) TA12:

Tryptone	...	...	10.0 g/l
Sodium chlorice	...	...	5.0 g/l
Agar-agar	...	...	12.0 g/l

(5) M9 Medium: The composition of M9 medium is as under:

Casamino acid	...	...	15.0 g/l
NaCl	...	...	0.59 g/l
NH_4Cl	...	...	0.963 g/l
Na_2HPO_4	...	...	6.5 g/l
KH_2PO_4	...	...	3.0 g/l
$MgSO_4.7H_2O$	...	...	0.62 g/l
Glucose	...	...	2.0 g/l

(6) 1XA medium. The medium used for the β-galactosidase assay had the following composition:

$K_2HPO_4.3H_2O$	...	...	10.5 g/l
KH_2PO_4	...	...	4.5 g/l
$(NH_4)_2SO_4$	...	...	1.0 g/l
Sodium citrate	...	...	0.5 g/l

After autoclave, the following sterilized solutions were added:

1 ml of $MgSO_4$ (1M)

*10 ml of glycerol (20%; v/v)

1 µg/ml thiamin

40 µg/ml each of the essential amino acids required for growth of the bacterium.

*For the construction of AB1157leu^+ from leu^- strain, the same constituents were used but with only one exception that in place of glycerol, 0.1% (w/v) lactose was added. This medium was solidified on plates by 1.8% (w/v) agar-agar.

(f) Buffer:

(1) Tris-Mg buffer (pH 8.0, 0.01M).

0.01M tris(hydroxy methyl) methyl amine			1.211 g/l
0.01M $MgSO_4.7H_2O$	...	...	2.465 g/l

pH was adjusted to 8.0

(2) $MgSO_4$ (0.01M). For all dilutions except otherwise stated, 0.01M $MgSO_4$ solution was used.

(3) Z-buffer. For β-galactosidase activity:

$Na_2HPO_4.7H_2O$	...	...	0.600M
$NaH_2PO_4.H_2O$	...	...	0.040M
KCl	...	...	0.010M
$MgSO_4.7H_2O$	...	...	0.001M
β-mercaptoethanol	...	...	0.050M

pH was adjusted to 7.0

(This buffer was not autoclaved)

(g) Composition of scintillation fluid:

PPO	...	4 g
POPOP	...	100 mg
Toluene (redistilled)	...	1 litre

10 ml of the scintillation fluid was used for every vial.

(h) **Chemicals**

The following chemicals were used:

Chemical	Source
Actinomycin D	Sigma Chemical Co., USA
^{3}H-actinomycin D	Amersham, England
Adenosine	Sigma, USA
Agar-agar	BDH
Ampicillin	Synthesized at our Institute (CDRI, Lucknow)
β-mercaptoethanol	BDH
Blue-dextran	Pharmacia, Fine Chemicals, Sweden
Bovine pancreatic DNase	Sigma, USA
Bovine pancreatic RNase	Sigma, USA
Bovine serum albumin	Sigma, USA
Calf thymus DNA	Sigma, USA
Casamino acid	Oxoid Limited, London
Chloramphenicol	A kind gift from May & Baker, India Ltd.
Chloroform	BDH
Essential amino acids	Centron, Research Laboratories, India
EDTA	BDH (Analar)

Chemical	Source
Glycerol	BDH
IPTG	A kind gift from Dr. Ranjana Srivastava
KCN	BDH
Lactose	BDH (Analar)
^{14}C-leucine	Bhabha Atomic Research Centre, Bombay, India
Lysozyme	Sigma, USA (3x crystallized)
Mitomycin C	Calbiochem, Switzerland
ONPG	A kind gift from Dr. Ranjana Srivastava
Polyethylene glycol (PEG, 6,000)	Belgolabo, Belgium
POPOP	V.P. Chest Institute, New Delhi, India
PPO	V.P.Chest Institute, New Delhi, India
Rifampicin	A kind gift from Prof. G.C. Lancini
Sephadex G-100	Pharmacia Fine Chemicals, Sweden
Sodium chloride	Sarabhai M.Chemicals, India (GR-grade)
Streptomycin	IDPL, India
TCA	Centron, Research Laboratories, India
^{3}H-thymidine	Bhabha Atomic Research Centre, Bombay, India
^{14}C-thymine	Bhabha Atomic Research Centre, Bombay, India
Toluene	BDH
Tris-HCl	BDH
Tryptone	Oxoid Limited, London

Chemical	Source
^{3}H-uridine	Bhabha Atomic Research Centre, Bombay, India
Vitamin B_1 (thiamin)	BDH (analar)
Yeast extract paste	Centron, Research Laboratories, India
Yeast RNA	Sigma, USA

Note: The chemicals, which are not included in this list, were purchased from BDH.

CHAPTER III

PURIFICATION OF BACTERIOPHAGE λ BY GEL FILTRATION

INTRODUCTION

Stocks of bacteriophage λ obtained by lytic growth in a sensitive host bacterium contain constituents of growth medium, the bacterial DNA, RNA, and other cellular components. For biochemical, genetical and radiobiological studies, it is necessary that the phage must be free from extraneous materials including host DNA and RNA.

Purification of viruses usually involves a series of fractionation procedures, necessarily performed under mild conditions to prevent destruction of functional structure of viruses. The most frequently used method of phage purification employ either differential centrifugation or caesium chloride (CsCl) gradient centrifugation followed by removal of CsCl from the phage fraction (Kaiser and Hogness, 1960; Yamamoto et al., 1970; De Ley et al., 1972). Though the method is simple, it is very expensive and not suitable in countries like India in view of the cost and maintenance of ultracentrifuge, nitrocellulose tubes and CsCl etc. The other method using hydroxyapatite chromatography was described by Miyazawa and Thomas (1965). Although, it does not involve the CsCl gradient step, but it allows most of the impurities to elute with phage λ . We, therefore, have developed a technique of phage purification by gel filtration which is efficient for bacteriophage λ , by passes the CsCl gradient step, and is relatively inexpensive and rapid. The principle of λ purification by gel filtration is simply based on the "Sieve theory" which implies that the molecular weight of one type of molecule (λ) is above the

exclusion range of the gel used, while the other type of molecules (all other constituents of stock other than λ) are capable of penetrating the gel.

MATERIALS AND METHODS

Phage strains: λ vir, λ*sus*1857, λ*b*2, λ*c*$_{I}^{ts}$, λ*red*, λ*R*^{90} and λ *bio*1 (described in Chapter II) were purified by this technique.

(a) Preparation of phage lysate: The crude lysate was made either by confluent lysis of C600 strain on nutrient agar plates or by lysis of infected bacteria in shaken tryptone broth (Adams, 1959; Miller, 1972; Chapter II). Heavy particles were removed by centrifugation at 5,000 r.p.m. at 4°C for 15 min. 2 ml of the crude lysate was applied on the Sephadex column to check the efficiency of column for the purification of crude stock. The remaining lysate was taken for nuclease treatment.

(b) Nuclease treatment: The crude lysate was first treated with bovine pancreatic RNase (10 μg/ml) for 30 min at 42°C with moderate shaking. The fibrous particles were centrifuged at 5,000 r.p.m. for 10 min and the supernatant was then treated with pancreatic DNase (10 μg/ml) in presence of 0.01M Mg^{2+} for 30 min at 37°C with constant shaking. The lysate was again centrifuged at 5,000 r.p.m. for 10 min to remove fibrous particles. At this step, again 2 ml fraction was loaded on the

Sephadex column for purification and the remaining lysate was further treated with polyethylene glycol (PEG).

(c) PEG-treatment: The volume of nuclease treated phage lysate was measured and crystals of sodium chloride were added to give final concentration of 0.5M. Polyethylene glycol (10% w/v; type 6000) was added to the lysate. The lysate containing NaCl and PEG was stirred at 4°C over a magnetic stirrer for at least 4 hr. PEG-treated phage lysate looked slightly opalescent. This was centrifuged in cold at 10,000 r.p.m. for 20 min. The supernatant was gently poured off and the pellet containing phage particles was suspended in 2 ml Tris-Mg buffer (0.01M, pH 8.0). Thus a 100 ml lysate had been reduced to 2 ml. The 2 ml buffer containing phage was centrifuged once at 2,000 r.p.m. for 5 min to remove heavy materials.

(d) Final Purification of phage: PEG-treated phage was finally purified by gel filtration using Sephadex G-100 at 10 to 20°C. The column specifications are as follows:

Diameter of the column	...	1.4 cm
Mass of dry gel used (approx)		2.4 g
Bed length	...	... 25.0 cm
Bed volume	...	... 38.3 ml
Void volume	...	... 13.8 ml
Flow rate	...	... 0.30 - 0.35 ml/min

Preparation and Use of Sephadex Column:

(a) Preparation of slurry: 3 g dry Sephadex G-100 was taken in

100 ml conical flask to which 60 ml distilled water was added and mixed with a glass rod. The flask was kept for 4 hr in a boiling water bath and then for 2 hr at room temperature for swelling and removing the air bubbles from the slurry. Sephadex was sedimented and the supernatant was discarded, and 10 ml fresh distilled water was added.

(b) Preparation of Sephadex column. A washed glass column was closed by a pinch cock, clamped vertically, and 10 ml distilled water was poured into the column. Wet piece of glass-wool was then cut out in the form of disc and put on the bottom of the column with the help of glass rod. Care was taken not to disturb the glass fibres, and to place it correctly. The glass rod was left pressing the glass fibre disc so that it does not move. All 10-15 ml slurry was gently poured down. After 10 min, the glass rod was gently taken out. The slurry was then poured upto the top with care that air bubble was not introduced. If air bubble appeared, it was removed by placing the tip of closed pipette near the bubble and then releasing the pressure of the pipette. When all slurry was poured down, the column was left closed for 2 to 3 hr for final setting. During the packing flow rate was kept identical to the working rate.

(c) Equilibration of column. For precision, accuracy and higher yield, it is necessary that pH and ionic strength of the Sephadex matrix (internal space of the Sephadex pores) must be identical to that of elution buffer. Therefore, elution buffer was passed through the column at the working flow rate till the pH of the buffer and effluent was the same. At this time the bed length of the column was also measured.

(d) Determination of void volume. Void volume was determined by loading 2 ml of blue-dextran (2.5 mg/ml) elution buffer on the column. The equilibrated column was taken and upper layer of elution buffer was removed by a pipette leaving behind approximately 2 ml buffer. The column was then opened and when just 2 ml buffer above the gel bed was about to be exhausted, it is closed and blue-dextran was very gently poured with a pipette. To have the uniform layering of blue-dextran, the tip of the pipette is uniformly moved in circular fashion. The column was then opened. The coloured layer moved down. When it reached the bed, 2 ml elution buffer was poured in circular fashion along the wall of column. The process was repeated twice and finally 10 ml buffer was poured. By this time, the coloured layer had moved down 5-10 cm below the top surface of the gel bed.

The volume of eluate was measured from the moment blue-dextran was applied till the coloured drop had come out from the column. This is the void volume. The column was re-equilibrated after determining the void volume.

(e) Purification of phage λ by Sephadex G-100 column. 2 ml phage stock was applied on the column and the elution was done by Tris-Mg buffer (0.01M, pH 8.0). The procedure is identical as described for void volume. Fractions of 2 ml each were collected, but in the vicinity of phage peak, the size of the fraction was reduced to 1 ml. To compare the degree of purification, crude stock, nuclease treated and nuclease-PEG

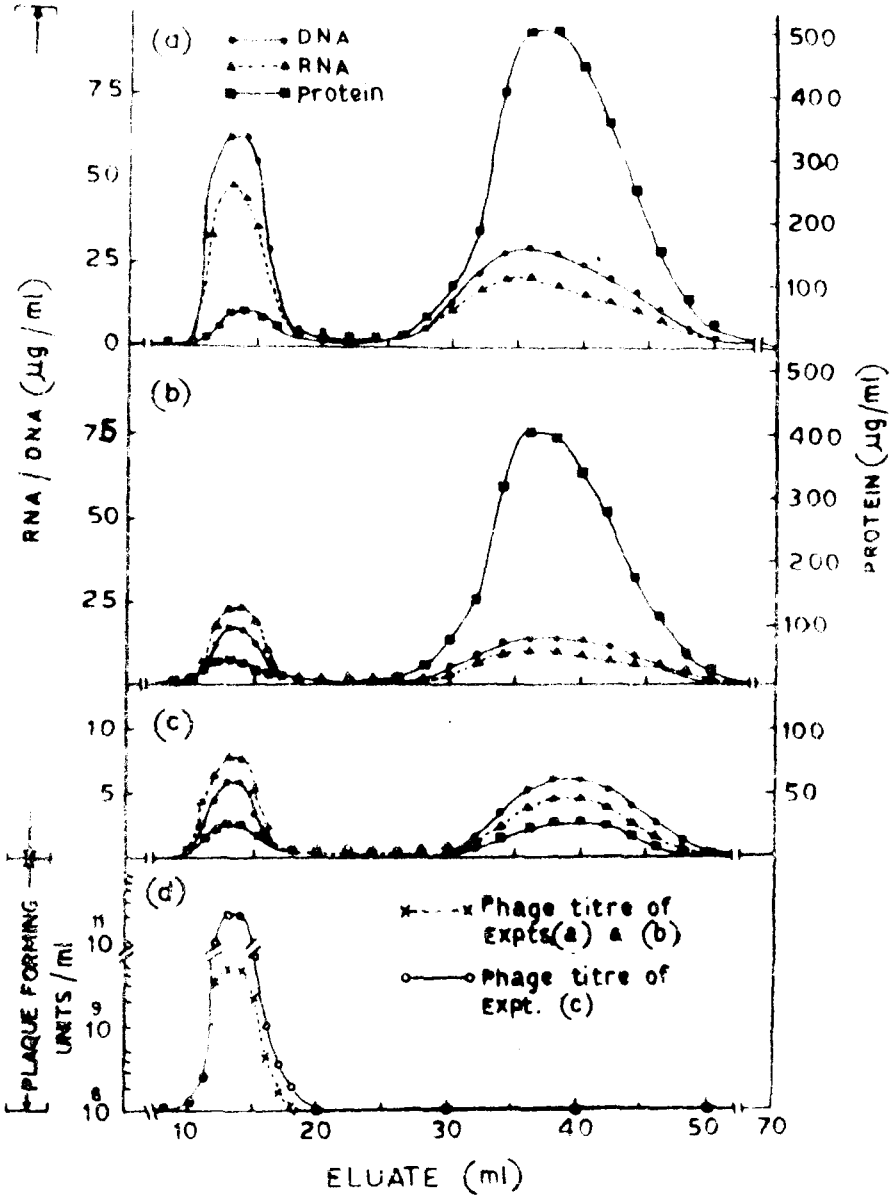
treated stocks were loaded and eluted through the column.

DNA, RNA and Protein Determinations of Eluted Fractions: Estimations of DNA, RNA and protein in each fraction were done according to the methods described by Burton (1956), Ceriotti (1955), and Loury et al. (1951) using calf thymus DNA, purified yeast RNA, and bovine serum albumin as standards respectively. Optical densities at 260 and 280 nm were also measured to follow the crude values of nucleic acid and protein in each fraction. Phage titre in each fraction was determined by plaque forming ability on C600 strain (Adams, 1959; Miller, 1972).

RESULTS

Typical elution profiles of DNA, RNA and protein for the purification of λ *vir* are shown in Fig. 1. Elution patterns of the crude, nuclease treated, and nuclease-PEG treated stocks loaded on the Sephadex column are respectively shown in (a), (b) and (c) compartments of Fig. 1. Fig. 1d shows the elution of phage λ during gel filtration of the three stocks. Invariably 98% of the phage titre was recovered from the Sephadex column. Since the total phage particles were collected into 8 fractions of 1 ml each, the stock was eventually diluted 4 times. 92% of the total phage was recovered in 5 fractions of highest titre (at 12-16 ml of elution volume) with a dilution factor of only 2.5 (Fig. 1d).

Fig. 1. Gel chromatography of λ*vir* after various steps: (a) Crude stock (b) Nuclease treated stock (c) Nuclease-PEG treated stock (d) Elution of phage particles.


(a)
DNA
RNA
Protein
(b)
(c)
(d)
Phage titre of expts(a) & (b)
Phage titre of expt. (c)
RNA / DNA (µg / ml)
PROTEIN (µg/ml)
PLAQUE FORMING UNITS / ml
ELUATE (ml)

It can be seen from Table 1 that crude lysate applied on the column contained 8.0, 0.60 and 0.45 mg of total protein, DNA and RNA respectively. But the phage peak contained only 0.2 mg of protein, 0.34 mg DNA, and 0.24 mg RNA. Thus 97.5% of proteins, 43% DNA and 46% RNA were removed from the crude lysate as a result of gel filtration.

When the stock is treated with nucleases, a significant drop in protein, DNA and RNA was observed (Table 1), nevertheless, the column removes 96.8% of proteins, 65% DNA and 40% RNA from the phage fractions.

Nuclease-PEG treated stock showed a sudden decrease in the protein, DNA and RNA as compared to nuclease treated stock (Table 1). The column removed 77% of the remaining proteins, 66% DNA and 56% RNA during the gel filtration.

The Table 1 also shows that minimum quantities of protein, DNA and RNA were present in the purified phage fraction with nuclease-PEG treated stock. Moreover, during the whole procedure these components reduced to 114, 20 and 13.5 folds respectively, as compared to the initial crude stock.

DISCUSSION

It is obvious from Fig. 1d that purified phage particles were eluted in the void volume (14 ml approx), whereas most of the impurities which include bacterial DNA, RNA and

Table 1. Purification Data of λ *vir* Applied on Sephadex Column

Steps	Total 'applied' phage stock contained in mg			Total 'eluted' phage stock contained in mg			Phage titre of applied stock
	Protein	DNA	RNA	Protein	DNA	RNA	
Crude lysate	8.0	0.6	0.45	0.2	0.34	0.24	7.4×10^{9}
Nuclease treated	4.5	0.23	0.20	0.15	0.08	0.12	7.3×10^{9}
Nuclease-DEC treated	0.3	0.09	0.08	0.07	0.03	0.035	2.8×10^{11}

protein, and other molecules of low molecular weight were eluted in the fractions quite different from those of the phage (Fig. 1). The separation volume between phage and impurities was approximately 20 ml for the column. It is also clear from Fig. 1 and Table 1 that DNA, RNA and protein contents of the stocks were decreased as we proceeded for the next steps of purification, nonetheless Sephadex G-100 column was capable of differentiating the phage and impurities at each step even from nuclease-PEG treated stock.

The values of RNA in the finally purified phage fractions are not absolute and precise due to the vicinity of lowest limit of colour detection. Furthermore, orcinol reagent imparts a little colour also with DNA, protein and agarose (Sephadex) which led to the overestimation of RNA content (Ceriotti, 1955). Folin's reagent may also interfere to some extent with DNA, RNA and agarose. It has also been reported that diphenylamine reagent interfere with RNA, proteins and agarose (Burton, 1956). As far as the presence of agarose in the phage fraction is concerned, it is well established that a little amount of Sephadex dissolves during elution. In view of this, the quantity of DNA in the purified phage ($2 \times 2.8 \times 10^{11}$ plaque forming units/ml) comes out to be 0.028 mg* which is comparable

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*Mass of $2 \times 2.8 \times 10^{11}$ phage particles was calculated according to the following formula:

$$\text{Mass of } \lambda \text{ in grams} = \frac{\text{Molecular weight of } \lambda \times \text{no. of } \lambda \text{ particles}}{\text{Avagadro's number}}$$

Contd.....

with the experimental values. Therefore, it may be concluded that phage purified from this technique did not contain any significant amount of extraneous DNA, RNA and protein.

Since the molecular weight of PEG, 6,000 does not exceed the exclusion limit of Sephadex G-100, PEG contamination in the nuclease-PEG treated stock is also ultimately removed during the gel filtration and thus the technique also by passes the step of dialysis before loading on the column.

The technique is very handy, inexpensive, rapid, and efficient for the purification of bacteriophage λ . There was no significant loss of viable phage particles during the elution through Sephadex column. Owing to low molecular weight of nucleases, there is no possibility of their contamination in phage fraction. Furthermore the technique can be employed for large scale purification of phage λ by recycling the PEG step. For that purpose, large amount (say 10 litre) of lysate can be concentrated 100 times by PEG treatment by dissolving the pellet in fresh medium. It will then be treated with nucleases and PEG followed by gel filtration. Care should be taken about 'holding capacity' of the Sephadex column. By holding capacity of the column we mean the maximum number of molecules which can freely interact to the Sephadex beads without any detectable hindrance. As one increases the amount of sample, the

---

Contd.....

$$= \frac{3 \times 10^{7} \times 2 \times 2.8 \times 10^{11}}{6.0 \times 10^{23}} \text{ g}$$

$$= \frac{16.8}{6.0} \times 10^{-5} \text{ g} = 0.028 \text{ mg}$$

effective interaction of the particle with the Sephadex will be less so that more of the impurities will come with phage λ . For our column dimensions, the maximum permissible amount of phage particles to be loaded is 10 mg of λ . Hence, a phage titre of 10^{14} plaque forming units/ml, obtained through repeated PEG treatments can be applied on the Sephadex column for their purification in a single gel filtration step.

In principle, the method is not only useful for the purification of phage λ but it should be equally applicable for other phages and simple viruses.

CHAPTER IV

EFFECT OF 52°C EXPOSURE ON *E.coli* K-12 : SURVIVAL, LIQUID HOLDING RECOVERY AND MUTAGENESIS

INTRODUCTION

Injury to bacteria caused by exposure to super optimal temperature and their subsequent recovery in *Staphylococcus aureus*, *Aerobacter aerogenes*, *Vibrio marinus*, *Streptococcus faecalis*, *Salmonella typhimurium*, *Vibrio haemolyticus*, *Bacillus subtilis*, *Pseudomonas fluorescence* and *Clostridium botulinum* involved alterations in their structural, physiological, permeability and biosynthetic characteristics (Busta and Jezeski, 1963; Strange and Shen, 1964; Iandolo and Ordal, 1966; Haight and Morita, 1966; Clark *et al.*, 1968; Clark and Ordal, 1969; Tomlins *et al.*, 1971, 1972; Miller and Ordal, 1972; Gray *et al.*, 1973; Pierson *et al.*, 1974; Gomez *et al.*, 1976). The heat injuries have been characterized as damage to the cell membrane, degradation of ribosomal RNA and alteration in enzyme activity (see reviews: Strange, 1976; Busta, 1976; Hurst, 1977). Bridges *et al.* (1969) demonstrated that mutation in certain loci, which render *E.coli* sensitive to radiation, also enhance sensitivity to heat. Heat treatment to *Escherichia coli* at 52°C apparently induces single-strand breaks in the DNA, and post-treatment incubation in phosphate buffer leads to the rejoining of breaks and increase in survival (Bridges *et al.*, 1969; Woodcock and Grigg, 1972). Incubation of UV-irradiated *E.coli* in minimal medium results in enhanced survival (Roberts and Aldous, 1949; Ganesan and Smith, 1958). Gomez *et al.*(1973) reported the minimal medium recovery of heated *S.typhimurium*. Heat exposure is also known to induce mutations in bacteria and

bacteriophage (Zamenhof and Greer, 1958; Zamenhof, 1960; Freese, 1961, Drake and McGuire, 1967; Baltz et al., 1976; Bingham et al., 1976). The work reported here was undertaken in an attempt to understand the nature of heat lesions in E.coli K-12, the requirements for recovery and involvement of known genetic loci.

MATERIALS AND METHODS

Media: Minimal and rich medium (or 869 medium), abbreviated as MM and RM respectively, have been used and are described in chapter II. We have frequently used the word 'recovery medium' which is the 869 medium. Viable counts of bacteria were always taken on RM solidified with 1.5% (w/v) agar.

Buffer: All dilutions of bacteria were made in 0.01M $MgSO_4$ solution. Bacteria held in Tris-Mg buffer (0.01M, pH8.0) were exposed to heat treatment.

Bacteria and growth condition: The following bacterial strains have been employed in this study: AB1157, AB2463, AB2494, AB1886, AB2470, AB2480 and JG112. The relevant genetic markers associated with these strains have been described in chapter II. Bacterial strains were maintained on tryptone agar slants at 4°C and routinely transferred every month. Overnight culture was grown at 37°C in MM or RM, as the case may be and diluted 50-times in fresh respective liquid medium. The cultures were shaken at 37°C till the cell number increased to about 2.5×10^8/ml.

Heat treatment: Bacteria, from an exponentially growing culture, were centrifuged, washed and resuspended in Tris-Mg buffer. The bacterial suspension (about 2 x 10^8/ml) was taken in a thin test tube and placed in a water bath kept at 52°C. The temperature in the tube rose to within 0.5°C of the final temperature in 1 min. The samples of bacteria were withdrawn at regular intervals, suitably diluted and plated to assay colony forming ability at 37°C.

Post-treatment incubation: Instead of immediate plating, heated bacteria were incubated in different conditions. Heated and unheated bacterial suspensions (0.5 ml) were diluted 10-fold into MM or RM and shaken at 37°C for 2 hr. During the 2 hr incubation, samples were taken out at intervals, diluted and plated. As described in the results of this chapter, incubation of heated bacteria in RM caused significant recovery. The effect of chloramphenicol and rifampicin was examined on recovery. For this, chloramphenicol (100 μg/ml) or rifampicin (10 μg/ml) was added in the medium. Chloramphenicol and rifampicin at these concentrations did not affect the viability of unheated bacteria. Before plating, bacteria were washed to remove the drugs.

Salt tolerance: The high salt medium consisted of RM containing 2% (w/v) sodium chloride on which *E.coli* grows with no detectable loss of viability. Bacteria were heated as described above and either plated directly on to the high salt medium or held in RM for 2 hr and then plated on high salt medium. The visible colonies growing at 37°C were counted.

Exposure to ultraviolet light(UV): Unheated, heated and heated bacteria held in recovery medium for 2 hr with and without chloramphenicol were compared for UV sensitivity. The source of UV was a Philips germicidal lamp emitting mainly at 254 nm. Unheated or heated bacteria after suitable dilutions were plated on RM and exposed to UV. Photoreactivation was avoided and plates were incubated at 37°C till visible colonies appeared.

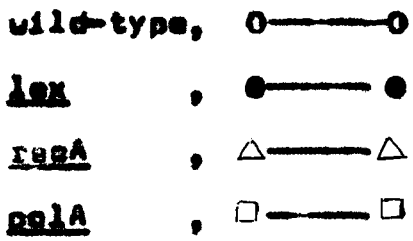
Sensitivity to mitomycin C: Sensitivity to mitomycin C was determined by plating the bacteria on RM containing the drug at 0.5 μg/ml. Mitomycin C at this concentration had no effect on the viability of unheated bacteria.

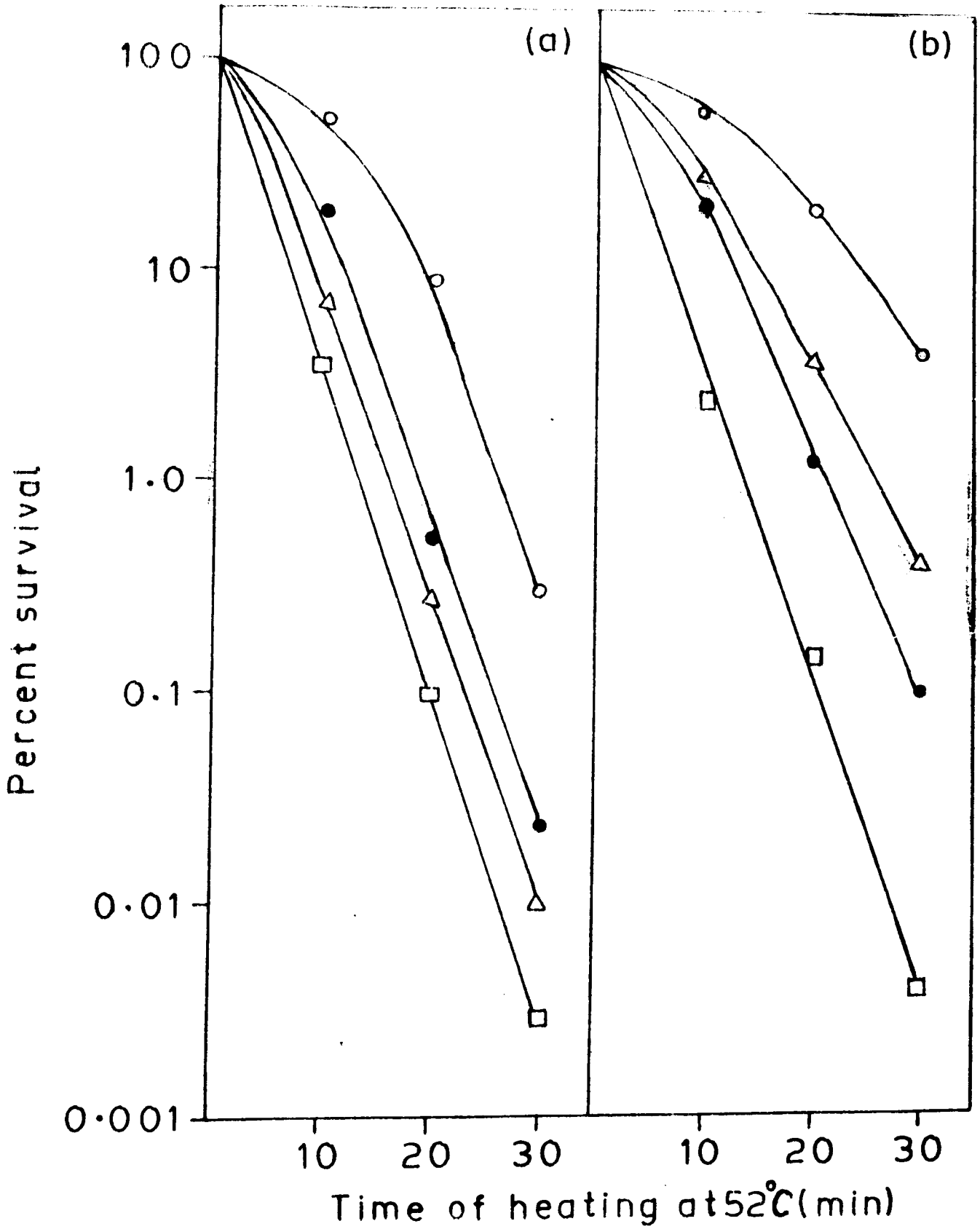
Heat-induced mutagenesis: Bacteria were heated and plated directly on tryptone agar containing rifampicin (100 μg/ml) or ampicillin (10 μg/ml) and incubated at 37°C to score *rif*^{r} (rifampicin-resistant) and *amp*^{r} (ampicillin-resistant) colonies. Heated bacteria held in recovery medium for 2 hr were also plated on media containing rifampicin and ampicillin.

RESULTS

Survival of heated bacteria: Bacteria were either grown in RM or RM before heat treatment and always plated on RM after heat treatment. The survival of bacteria plated directly on to tryptone agar after heating is shown in Fig. 1. Inactivation

Fig. 1. Survival of bacteria exposed to 52°C.
(a) Pregrown in MM (b) Pregrown in RM.




(a)
(b)
100
10
1·0
0·1
0·01
0·001
Percent survival
10
20
30
10
20
30
Time of heating at 52°C (min)

of colony forming ability was observed after heat treatment which increased with increase in exposure to heat. The mutants recA, lex and polA were more sensitive to heat than the wild-type. Mutants uvrA and recB were as resistant as the parent strain and uvrA⁻ recA double mutant was as heat sensitive as the recA alone (data not shown). The wild-type and recA strains were more sensitive when grown in MM than when grown in MM before heat treatment. However, viability of polA, and to a large extent lex, was not affected by pre-heat treatment growth medium.

Liquid holding recovery in MM and MM: The bacteria, grown in MM or MM were heated for 30 min at 52°C and then shaken for 2 hr in MM or MM at 37°C. Colony-forming ability was assayed at intervals during this period by plating samples on tryptone agar. The viability of unheated bacteria under the same conditions was also assayed (Fig. 2). Recovery was influenced by the composition of both the pre- and post-treatment media. Recovery is the ratio of the viable numbers of heated and unheated bacteria at 2 hr.

The results shown in Fig. 2 (a,c) are for bacteria grown in MM and MM respectively, and shaken in MM after heat treatment. When grown in MM before heat treatment, the wild-type and recA strains showed recovery during the 2 hr incubation, whereas lex and polA mutants did not (Fig.2a). When grown in MM before treatment, the colony forming ability of recA and lex declined, whereas with the wild-type loss of viability occurred for 1 hr (Fig. 2a). Again, polA did not recover.

Fig. 2. Recovery of 30 min heated bacteria.

(a) Pregrown in MM and incubated in MM
(b) Pregrown in MM and incubated in RM
(c) Pregrown in RM and incubated in MM
(d) Pregrown in RM and incubated in RM

Viable counts of heated cells, (———)

Increase in the viable counts of unheated cell under similar conditions, (— — —)

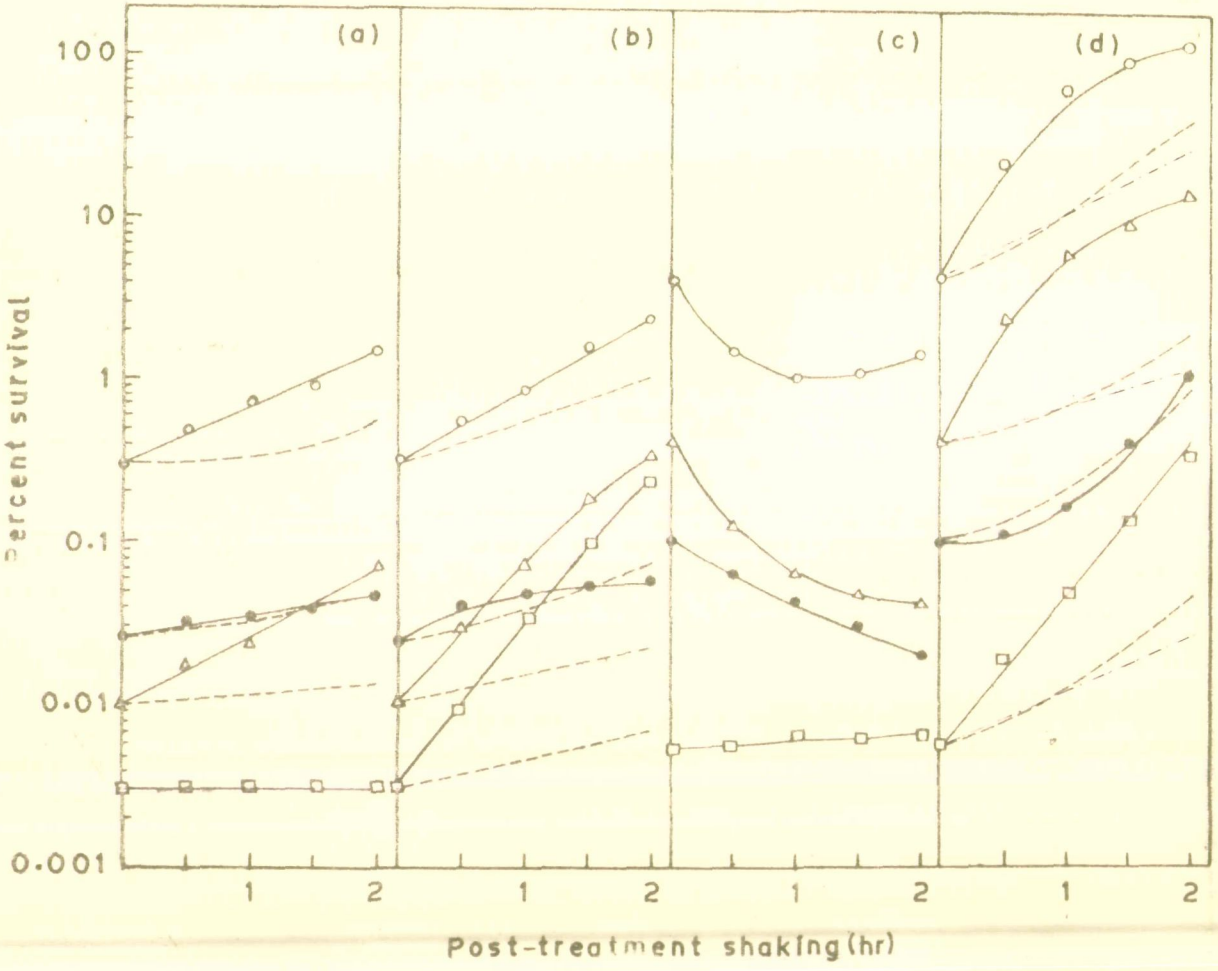
The number of viable counts in the presence of chloramphenicol, (-·-·-·-)

wild-type, O———O

lex , ●———●

recA , △———△

polA , □———□


(a)
(b)
(c)
(d)
100
10
1
0.1
0.01
0.001
Percent survival
1
2
1
2
1
2
1
2
Post-treatment shaking (hr)

The results shown in Fig. 2(b,d) are for bacteria grown in MM and MM respectively and shaken in MM after heat treatment. Except for the *lex* mutant, all the strains recovered significantly. The apparent extent of recovery after 2 hr for the wild-type, *recA*, *polA* and *lex* strains was 3, 7, 10 and 1 respectively (Fig. 2d). However, the *lex* mutant failed to recover and the increase in viable counts of the *lex* mutant was probably due to the multiplication of survivors since under identical conditions the increase in the number of unheated bacteria was the same.

When bacteria grown and treated as in Fig. 2(d) were not shaken but simply held in tryptone broth, the same results were obtained. Thus, holding in tryptone broth for 2 hr after heat treatment increased survival compared with immediate plating on tryptone agar. Tryptone broth can therefore be referred to as recovery medium.

For convenience, we have interpreted the recovery in terms of ratio of viable counts of heated and unheated cells at 2 hr of liquid-holding (as described above). But the absolute number of recovered cells during the liquid-holding could be calculated according to the equation (3) of the appendix. These values are given in Table 1. Table 1 is based on the assumptions made in the appendix for the combination MM-MM (Fig. 2d). Although, the strains *polA* and *lex* did not follow the equation (3), but the minimum number of recovered cells can be obtained from this equation. Recovery pattern of

Table 1. Recovery of heated cells during post-treatment incubation in RM. The initial number of cells subjected to 52°C was 10^8/ml.

Strains	Initial viable counts ml^{-1} of bacteria after 30 min heat treatment	No. of cells recovered during post-treatment incubation*			Fold recovery at 120 min
		30 min	60 min	120 min	
Wild-type	4×10^6	1.2×10^7	2.4×10^7	4.8×10^7	12
recA	4×10^5	1.5×10^5	3.0×10^5	6.0×10^6	15
lex	1×10^5	Zero	Zero	3.0×10^4	0.33
polA	5×10^3	4.6×10^3	2.0×10^4	1.0×10^5	20

*See appendix

heated wild-type and recA strains in fig.2d strictly followed the equation (3). The Table 1 showed that, though w.t.(wild-type), recA, and polA recovered during the first hour of liquid Holding, lexA could not exhibit this recovery. The fold-recovery for heated w.t., recA, polA and lex strains after 2 hr was 12, 15, 20 and 0.33 respectively (Table 1).

Recovery of high salt tolerance, mitomycin C and UV sensitivity during liquid holding: When heated bacteria were plated on high salt medium, the number of bacteria capable of making colonies was less than on the tryptone agar alone. Heated bacteria held in recovery medium for 120 min showed increased colony forming ability on high salt medium and there was little difference between colony forming ability of bacteria on high salt medium and plain tryptone agar (Table 2).

Similar results were obtained on plating efficiency of bacteria on mitomycin C containing plates. Heated bacteria were more sensitive to plating on mitomycin C media. 2 hr incubation of heated bacteria in recovery medium resulted in resumption of normal mitomycin C resistance (Table 2).

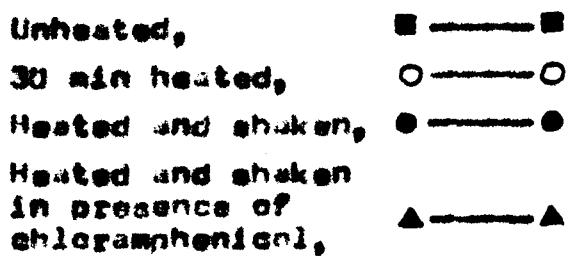
UV sensitivity of unheated, 30 min heated bacteria, and heated bacteria held for 2 hr at 37°C in recovery medium was examined. Unheated AB1157 showed normal resistance to UV light whereas 30 min heated AB1157 was comparatively sensitive to UV killing. When 30 min heated bacteria were held in recovery medium and then exposed to UV, normal UV resistance of bacteria comparable to unheated control was observed (fig. 3).

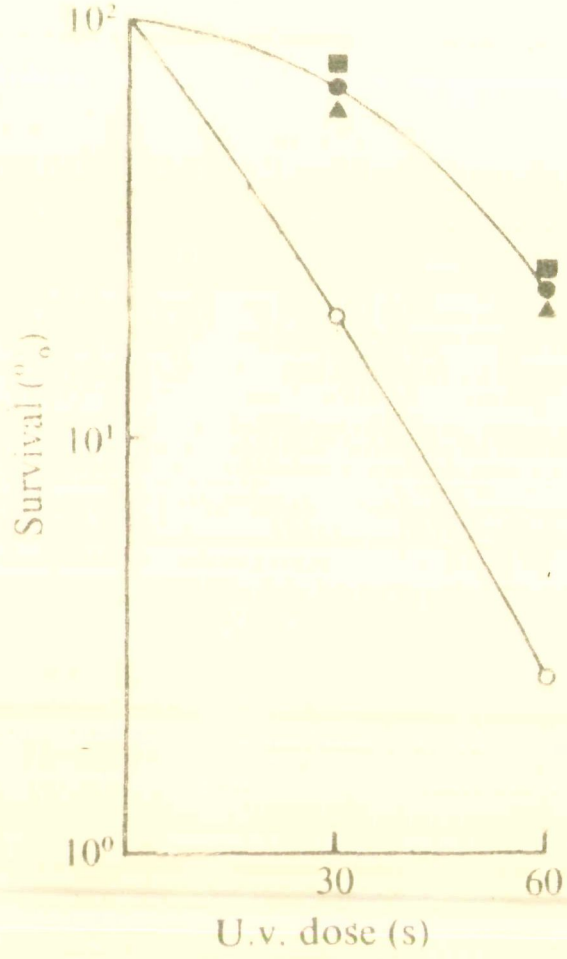
Table 2. Effect of metabolic inhibitors on the liquid holding recovery of heated AB1157

Bacteria	Incubation medium	Incubation time (min)	Viable units ml^{-1} on different plating media		
			RM	RM + NaCl (2%)	RM+mitomycin C
Unheated AB1157	RM	0	2.0×10^7	2.2×10^7	2.0×10^7
	RM	120	1.4×10^7	ND	ND
	RM+Rif	120	1.2×10^7	ND	ND
	RM+Cam	120	2.0×10^7	ND	ND
Heated AB1157 (30 min, 52°C)	RM	0	4.3×10^5	3.5×10^4	3.5×10^4
	RM	120	6.5×10^6	1.2×10^6	4.0×10^6
	RM+Rif	120	4.5×10^4	2.2×10^5	3.0×10^3
	RM+Cam	120	1.2×10^6	2.5×10^5	3.3×10^5

ND, not done; Cam, chloramphenicol; Rif, rifampicin.

Fig. 3. UV sensitivity of AB1157 under different conditions:




10^2
10^1
10^0
Survival (%)
30
60
U.v. dose (s)

Effect of rifampicin and chloramphenicol on liquid holding recovery: Both the antibiotics at their respective concentrations did not cause lethality of unheated bacteria.

When heated bacteria were held in recovery medium containing rifampicin, recovery of survival was considerably inhibited. The drug also interferred with the recovery of mitomycin C resistance. Recovery of high salt tolerance was also affected by rifampicin but the degree of inhibition was less compared to mitomycin C resistance (Table 2).

The presence of chloramphenicol in the recovery medium during 2 hr incubation of heated bacteria inhibited the recovery of viability. However, some recovery did occur even in the presence of chloramphenicol (Table 2, Fig. 2d). Chloramphenicol interferred to a large extent with the recovery of salt tolerance and mitomycin C resistance (Table 2), but it had no effect on the recovery of UV resistance (Fig. 3).

Heat-induced mutagenesis: Heat-induced mutations to rifampicin and ampicillin resistance was scored and mutation frequency is given in Table 3. Each figure is an average of three experiments done in triplicate. It was found that 52°C heat treatment is a poor mutagen. An increase in the number of amp^r and rif^r colonies was observed following heat-treatment to AB1157(Table 3).

When heated AB1157 was held in the recovery medium for 2 hr and then plated to score amp^r and rif^r colonies, loss of mutagenesis was observed (Table 3).

Table 3. Heat mutagenesis in AB1157.

Heat Treatment (min)	Mutation Frequency			
	$\underline{amp}^r$ (per 10^8 Viable Counts)		$\underline{rif}^r$ (per 10^8 Viable Counts)	
	Without liquid holding	Held in recovery medium for 2 hr	Without liquid holding	Held in recovery medium for 2 hr
0	1.4	1.2	4.6	4.3
15	2.2	1.3	12	5.0
30	6.0	1.9	19	7.4

DISCUSSION

Bridges et al. (1969) found that 'reckless' and 'cautious' recombination-deficient strains of E.coli B were sensitive to mild heating whereas the exr (lex) mutation had little or no effect except when combined with a lon or hcr mutation. The heat sensitivity of polA$^+$ and polA strains was identical (Sedgwick and Bridges, 1972) and the authors explained this as being due to the lack of endonucleolytic incision activity. Part of our data on the apparent involvement of known loci on heat sensitivity do not agree with these results. We found that besides the recA strain, polA and lon strains were also sensitive to heat whereas the 'cautious' strain recB was as resistant as the wild-type. The 'cautious' recombination-deficient strain used by Bridges et al. (1969) was later shown to have a recA mutation (Kato and Kondo, 1970), which is an allele of polA. This might explain why the 'cautious' recombination deficient strain of Bridges et al. (1969) was sensitive to heat killing.

Survival was enhanced when the heated bacteria were held in tryptone broth as compared to direct plating on tryptone agar. The effect of 'liquid holding' on the recovery of UV irradiated E.coli in MM is well documented (Roberts and Aldous, 1949; Ganesan and Smith, 1960). Contrary to the results with UV, the liquid holding recovery of heated bacteria was poor in MM compared with recovery in AM.

Recovery of viability was considerably inhibited by rifampicin and chloramphenicol, suggesting a possible role for

RNA and protein syntheses in recovery, Salmonella typhimurium also required both for recovery from thermal injury (Tomlins and Ordal, 1971), other bacteria do not require protein synthesis (Clark et al., 1968; Miller and Ordal, 1972; Gray et al., 1973).

Heated bacteria were more sensitive to UV and to plating on high salt medium, and during liquid holding normal UV resistance and salt tolerance were regained. These data suggest inactivation of radiation repair enzymes and damage to the cell membrane by heat and their subsequent recovery. The recovery of UV resistance in the presence of chloramphenicol (Fig. 3) suggests that de novo synthesis of repair enzymes are not required and under favourable conditions, provided by tryptone broth, renaturation of the repair enzymes might be involved. Chloramphenicol inhibited the recovery of mitomycin C resistance and salt tolerance (Table 2) which suggests that repair of the cell membrane requires de novo protein synthesis. Thus, the failure to achieve normal mitomycin C resistance in the presence of chloramphenicol could be due to increased permeability of mitomycin C. The recovery of normal UV resistance in the presence of chloramphenicol (Fig. 3) but inhibition of recovery of viable bacteria in recovery medium (Fig. 2d) suggest that the two phenomena may be independent of each other.

The repair of certain heat lesions occured during liquid holding and not on agar plates of the same medium composition. We suggest that lesions in protein and membrane are repaired in the liquid medium but inhibited on agar plates. A similar

result was reported by Roberts and Aldous (1949) who tests the inhibitory effect of agar on recovery of UV irradiated E.coli. Recovery was obtained in liquid medium but was inhibited on agar medium and they attributed this effect to a typical diffusion which was not possible on the solid medium.

Gray et al. (1973) have suggested that there was no multiplication of heated P.fluorescence during recovery in the growth medium. Even, if the multiplication of heated E.coli occurred under our experimental conditions, it was corrected by two different ways, i.e. (1) dividing the total increase of heated cells by the multiplication factor of heated cells during the liquid holding for 2 hr, and (2) by mathematical calculation (see appendix).

The calculation based on appendix is more appropriate and close to reality because, the heated cell would not divide unless fully repaired.

We have checked the validity of equation (3) of the appendix and found that this equation followed the same pattern of survival as the heated wild-type and recA showed in fig.2d. We, therefore, suggest that in this condition w.t. and recA recovered in an arithmetical progression (i.e. with constant rate) whereas polA recovered in geometrical progression (i.e. with increasing rate). The lex mutant was unable to recover upto 60 min of liquid holding.

Our data show that 52°C thermal treatment is a poor mutagen compared to UV. Dose of UV giving 50% survival of AB1157 yields higher number of ampicillin and rifampicin resistant colonies compared to 52°C-treatment giving the same survival. Liquid holding, which results in recovery of heated bacteria eliminated mutations to drug resistance suggesting that mutagenic lesions were repaired during liquid holding recovery.

APPENDIX

Mathematical interpretation of recovery

The following assumptions were made:

1. That the heated cells started to multiply immediately after the recovery from heat lesions.

2. That generation time of recovered cells was equal to that of unheated control cells in one particular medium.

3. That the rate of recovery remained constant throughout the incubation period.

4. That when the cells were seeded on plates immediately after treatment the recovery was negligible as compared to the recovery obtained during post-treatment incubation in aerated MM.

The considered:

N_o = Survival of heated cells without post-treatment incubation.

N = Survival of heated cells after post-treatment incubation at time T.

Tg = Generation time.

Δt = Time of incubation in which "one" cell recovered.

n = Number of recovered cells during incubation period T.

By 3rd assumption,

$$n.\Delta t = T \quad \ldots \quad \ldots \quad \ldots \quad (1)$$

The number of survivors N during incubation for time T would be given by the expression,

$$N = N_0.2^{n.\Delta t/Tg}+2^{(n-1).\Delta t/Tg}+2^{(n-2).\Delta t/Tg}+\ldots\ldots+2^{\Delta t/Tg}+1$$

which can be simplified as,

$$N = N_0.2^{T/Tg}+(2^{T/Tg}-1)/(2^{\Delta t/Tg}-1)$$

Substituting T/n for Δt and simplifying,

$$2^{T/n.Tg}-1+(2^{T/Tg}-1)/(N-N_0.2^{T/Tg})$$

$$\text{or, } n = \frac{T \times \log_{10}2}{Tg \times \log_{10}\left[1+\dfrac{(2^{T/Tg}-1)}{(N-N_0.2^{T/Tg})}\right]} \quad \ldots \quad (2)$$

Since value of $\dfrac{(2^{T/Tg}-1)}{(N-N_0.2^{T/Tg})}$ was experimentally much less than '1' (from 10^{-3} to 10^{-7}), and to avoid logarithmic expression, the equation (2) can be converted into the following equation,

$$n = \frac{\log_{10}2 \times T \times (N-N_0.2^{T/Tg})}{\log_{10}e \times Tg \times (2^{T/Tg}-1)}$$

$$\text{or} \quad n = \frac{0.30103 \times T \times (N-N_0.2^{T/Tg})}{0.43429 \times Tg \times (2^{T/Tg}-1)} \quad \ldots \quad (3)$$

The "absolute recovery" in the medium supporting recovery as well as growth was determined using equation (3).

Under the experimental conditions shown in Fig. 2d, wild-type and recA strains followed the equation (3), whereas lex and polA did not follow because assumption (3) was not being fulfilled. However, lower limit of recovery could be determined using equation (3).

Table 1 derived from equation (3) indicates that the number of cells recovered during the liquid holding was always less than the number of wild-type cells recovered during that period, despite the higher fold-recovery of recA and polA mutants.

CHAPTER V

BIOCHEMISTRY OF HEAT LESIONS INDUCED AT 52°C

INTRODUCTION

Considerable work has been done on biosynthetic processes involved in the recovery of heat-injured bacteria (see reviews: Gray and Postgate, 1976; Strange, 1976; Busta, 1976, Hurst, 1977).

Since heated cells were susceptible to plating on high salt medium and there was leakage of RNA, protein, and amino acids from heated cells, it was suggested that heat-treatment induces damage to cell membrane (Busta and Jezeski, 1963; Iandolo and Ordal, 1966; Sogin and Ordal, 1967; Kenis and Morita, 1968; Tomlins, 1969; Tomlins and Ordal, 1971b; Adea, 1973; Smolka et al., 1974). Heated cells showed altered uptake of glucose, degradation of RNA and loss or change in enzyme activity (Bluhm and Ordal, 1969; Rosenthal and Iandolo, 1970; Pierson and Ordal, 1971; Tomlins et al., 1971; Hurst et al., 1973).

Single and double strand breaks were not found when DNA was heated *in vitro* at mild-temperature (Inman, 1966). Strand scissions in the DNA of heated bacteria were observed *in vivo* (Bridges et al., 1969b; Woodcock and Grigg, 1972; Gomez and Sinskey, 1973), and these breaks were repairable (Mukherjee and Bhattacharjee, 1970; Woodcock and Grigg, 1972; Gomez and Sinskey, 1973; Gomez et al., 1976).

It appeared from the literature that protein synthesis

are not involved in the repair of heat-lesions of the bacteria namely Staphylococcus aureus, Streptococcus faecalis, Bacillus subtilis, Pseudomonas fluorescence whereas in Salmonella typhimurium and Vibrio parahaemolyticus protein synthesis was required (Clark et al., 1968; Bluhm and Ordal, 1969; Pierson et al., 1971; Tomlins and Ordal, 1971a; Miller and Ordal, 1972; Gray et al., 1973; Gomez et al., 1976; Heinis et al., 1978).

The involvement of RNA synthesis in the recovery of heat-damage has been reported for several bacteria (Iandolo and Ordal, 1966; Sogin and Ordal, 1967; Clark et al., 1968; Bluhm and Ordal, 1969; Pierson et al., 1971; Tomlins and Ordal, 1971 a,b; Rosenthol et al., 1972; Miller and Ordal, 1972; Gray et al., 1973; Gomez et al., 1976; Heinis et al., 1978).

In view of the literature mentioned above, it is concluded that different bacteria utilize different metabolic pathways for heat-repair and survival, and very little work has been done with Escherichia coli.

We have described in this chapter our results on the effect of heat on DNA strand-scission of E.coli and their repair; synthesis and degradation of DNA; leakage, synthesis and degradation of RNA; synthesis and degradation of protein, and permeability of heated cells.

MATERIALS AND METHODS

DNA degradation: Bacteria were labelled by growing exponentially in minimal medium supplemented with required growth factors and 5 μCi/ml ^{3}H-thymidine (specific activity, 6,500 mCi/m mole). The bacteria were grown upto 2 x 10^{8} viable counts/ml at 37°C. They were centrifuged and washed to remove labelled medium. Labelled bacteria were heated for 30 min at 52°C. After heat-treatment, cells were diluted 10-times and shaken in recovery medium at 37°C. 0.2 ml bacterial sample was taken out at regular intervals, fixed for 30 min in 1 ml chilled 5% (w/v) trichloroacetic acid (TCA). Acid insoluble fractions were collected on millipore filters (Millipore, USA; pore size, 0.45 μ). Radioactivity was counted in Packard Liquid Scintillation Counter. Unheated control bacteria were also processed in a similar manner.

← DNA synthesis: After heat-treatment in Tris-Mg buffer (0.01M, pH 8.0), the cell suspension was diluted 10-times in the recovery medium containing 0.4 μCi/ml ^{14}C-thymine (specific activity, 55.9 mCi/m mole). Unlabelled adenosine (100 μg/ml) was also supplemented to enhance the uptake of ^{14}C-thymine (Boyce and Setlow, 1962). The cell suspension was then incubated at 37°C with moderate shaking. 1 ml samples were taken out at regular intervals and fixed for 30 min in 2.0 ml 7.5% TCA. Acid insoluble fractions were obtained on millipore filters (Maxflow, India; pore size 0.45 μ) by filtration. The

material was washed with chilled 2.5% TCA and the radioactivity was measured in the liquid scintillation counter. Controls were also run accordingly.

Sucrose gradient centrifugation: Cells were grown exponentially in MM medium supplemented with required growth factors (40 µg/ml), 100 µg/ml adenosine and 15 µCi/ml ^{3}H-thymidine (specific activity 6,500 mCi/m mole). These cells were then grown in the same medium without tritiated thymidine for about one doubling to chase the label. Labelled cells were then centrifuged at low speed, washed twice and resuspended in 0.01M Tris-Mg buffer (pH 8.0) at a cell density of 2 x 10^{8}/ml. The labelled cells were divided into two portions. One was kept at 37°C which served as control whereas the other portion was treated at 52°C. 3.0 ml of the heated and unheated cell suspensions were centrifuged. Supernatant was discarded and the pellet of bacteria kept at ice temperature.

Spheroplasts were produced at ice-temperature by suspending the pellet in 0.3 ml B9 buffer plus 0.06 ml of a 30.0% (w/v) sucrose in 0.6 M Tris(pH 8.1). To this suspension were added 0.04 ml lysozyme (10% w/v solution) and 0.1 ml of 32 mM EDTA. After 5 min in ice, 10 µl of the spheroplast suspensions were placed on top of a 4.8 ml gradient of 5 to 20% (w/v) sucrose solution containing 0.1N NaOH, 0.9M NaCl and 10^{-3}M EDTA adjusted to pH 11.5 - 12.0. 0.1 ml spheroplasts and 0.01 ml marker ^{14}C-labelled λ 890 were mixed into a 0.1 ml top layer of 0.5 N NaOH by stirring several times

with a pin to release the DNA. The tubes were balanced and centrifuged at 30,000 r.p.m. for 120 min in SW39 rotor at 20°C in a Beckman L centrifuge. After centrifugation, nitrocellulose tubes were punctured and drops were collected on filter paper discs and counted in the liquid scintillation counter.

Preparation of labelled phage λ: Exponential culture of C600 was raised from overnight culture in fresh Mg medium. The cells were centrifuged and resuspended in Tris-Mg buffer to raise the cell density upto 10^9 viable counts/ml.

4×10^8 plaque-forming units (PFU)/ λ of cI90 was mixed with 10^9 cells/ml of C600 and adsorbed for 20 min at 37°C. The infective centres were shaken at 37°C in 20 ml M9 medium containing 50 μCi ^{14}C-thymine (specific activity 55.9 mCi/m mole). When mass lysis of bacteria was visible to the naked eyes, 1.0% chloroform was added. The lysate was centrifuged at 5,000 r.p.m. for 20 min to remove the debris etc., and the supernatant was treated with 10 μg/ml RNase at 42°C for 30 min followed by centrifugation at 5,000 r.p.m. for 20 min. The pellet was discarded and the supernatant was again treated with 10 μg/ml DNase at 37°C for 30 min in the presence of 0.01 M Mg^{2+}. Nuclease treated lysate was finally centrifuged at 6,000 r.p.m. for 20 min to remove fibrous material. Concentration of viral particles and their purification has been done according to the method described in chapter III.

RNA leakage during heat treatment: Exponentially growing bacteria were labelled with 4 μCi/ml ^{3}H-uridine (sp. activity, 10.9 Ci/m mole) as described above. Labelled cells (5×10^{8} viable counts/ml) were centrifuged, washed with treatment buffer (i.e. 0.01M Tris-Mg buffer, pH 8.0) and then suspended in the same buffer to raise the cell density around 2×10^{8} viable counts/ml. Inoculum was divided into two parts. First part was kept at 37°C and served as control. The other was further subdivided into three parts, each having 1.0 ml cell suspension, and separately treated at 52°C for 10, 20 and 30 min. The samples, after treatment, were centrifuged at 5,000 r.p.m. for 15 min. The supernatants were gently collected and counted for radioactivity.

RNA degradation during liquid-holding: Bacteria were labelled with ^{3}H-uridine as described above. The cells were centrifuged, washed twice to remove any free label, and resuspended in Tris-Mg buffer. The cell suspension was then divided into two equal parts. One part was kept at 37°C for control samples and the other part was treated at 52°C for 30 min. Treated samples were divided into two parts again. In one, cells were suspended in Tris-Mg buffer and the other part was suspended in RM. Both were held at 37°C and shaken at regular intervals. From heated as well as control flasks, 1 ml sample was taken out, fixed with 2.0 ml chilled TCA (7.5%). 0.1 ml bovine serum albumin (1% w/v) was also added to increase the volume of the precipitate. The samples were centrifuged in cold at 4,500 r.p.m.

for 20 min and the supernatants were gently collected on filter papers and counted in a liquid scintillation counter.

RNA synthesis: The bacteria were heated at 52°C for 30 min. Heated and unheated cells were diluted 10-times into recovery medium containing 4 μCi/ml ^{3}H-uridine (sp.activity 10,900 mCi/m mole) and 100 μg/ml unlabelled adenosine. The culture were then incubated at 37°C with moderate shaking in the metabolic shaker. Samples at suitable intervals were taken out, fixed with TCA and counted as described earlier.

Protein leakage, degradation and synthesis: The same procedure as described for RNA was also followed for protein. In place of ^{3}H-uridine, 0.2 μCi/ml ^{14}C-leucine (sp.activity 203 mCi/m mol) was supplemented to follow leakage, degradation and synthesis of protein in heated and unheated E.coli K-12.

Wild-type abbreviated as w.t. (AB1157), and lex (AB2494) strains were tested for these biochemical parameters. The latter was selected because of its inability to recover during liquid holding.

Permeability of cells to actinomycin D: Wild-type bacteria were grown exponentially upto 2 x 10^{8} cells/ml, centrifuged, washed and suspended in 0.01M Tris-Mg buffer (pH 8.0) and heated at 52°C. Two sets of experiments were done. In one case bacteria were heated for different time intervals. Heated samples were exposed for 10 min to ^{3}H-actinomycin D

(5 μCi/ml) and then chilled for 30 min. Pellet containing bacteria was obtained by repeated centrifugation in cold. During washing of cells, the buffer contained unlabelled actinomycin D to avoid leakage of labelled actinomycin D from inside of the cell. Radioactivity in the pellet was counted.

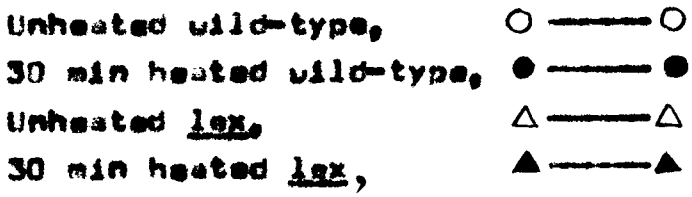
In the other case, bacteria were heated at 52°C for 30 min in Tris–Mg buffer and further incubated separately for 1 hr in Tris–Mg buffer and recovery medium. At intervals bacteria were pipetted out, incubated with actinomycin D for 10 min, and as described above washed pellets of bacteria were obtained and counted for radioactivity.

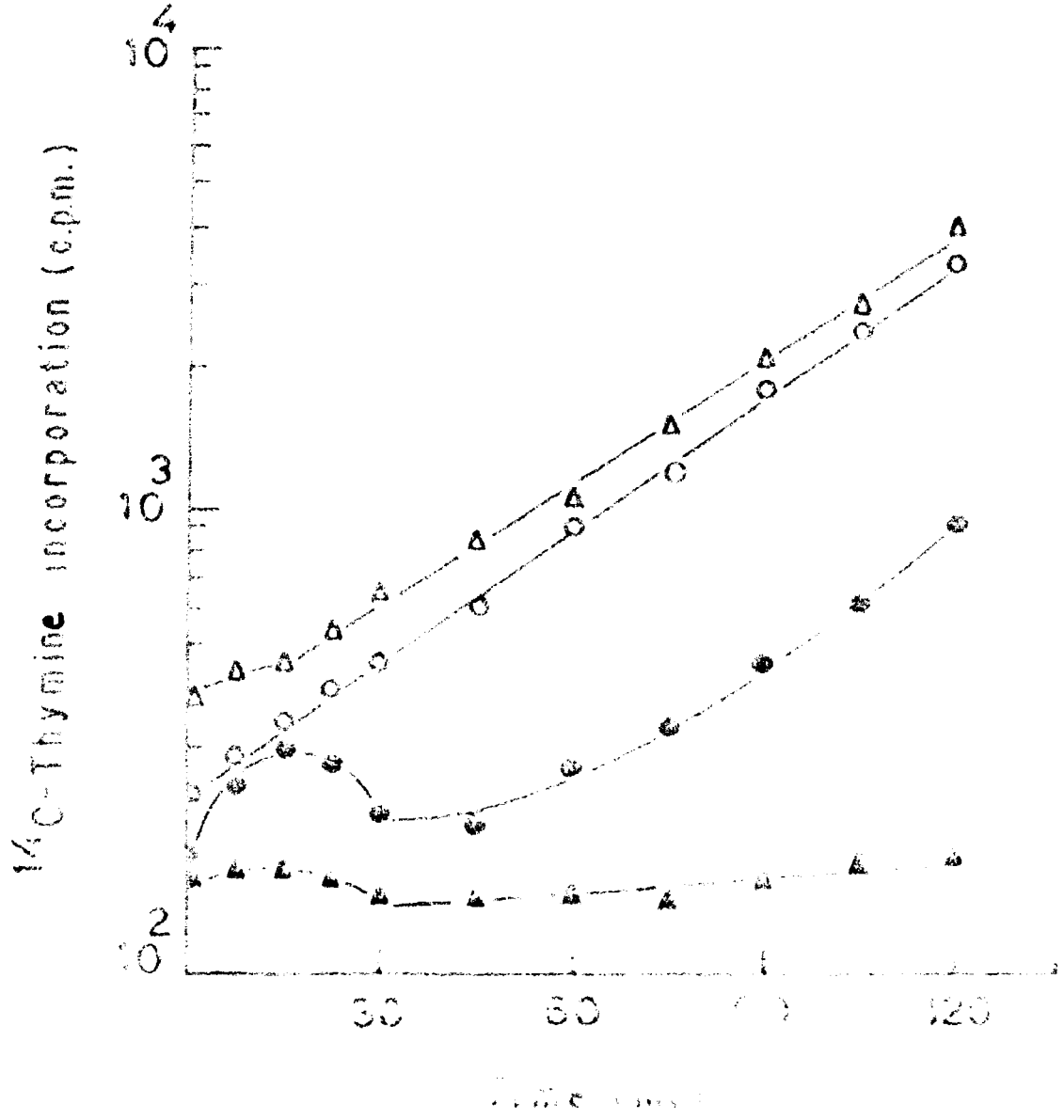
It is known that incubation of bacteria with EDTA enhances permeability (Leive, 1965). Therfore for comparison with heat-treatment, bacteria were held for 2 min at 37°C in triple distilled water containing EDTA (2×10^{-4}M). The reaction was stopped by adding 0.1M Mg^{2+} solution to make up the final concentration of Mg^{2+} to 0.01M. EDTA exposed cells were then exposed to 5 μCi ^{3}H-actinomycin D and radioactivity was counted as described above.

RESULTS

DNA synthesis: DNA synthesis was followed in unheated and heated cells of AB1157 and AB2494 and uptake of ^{14}C-thymine was followed (Fig. 1). Unheated bacteria incorporated

Fig. 1. DNA synthesis in wild-type and *lex* strains during incubation in recovery medium at 37°C.



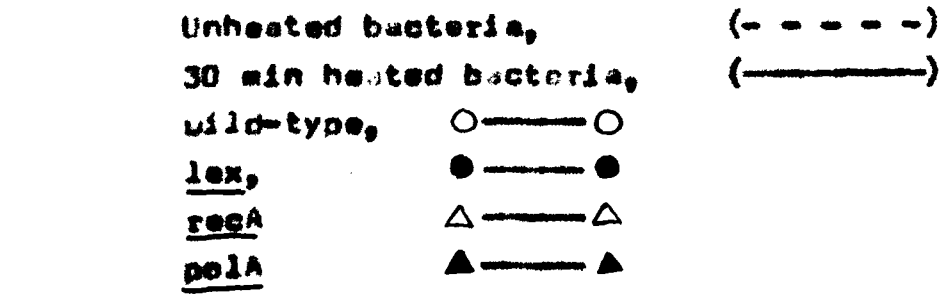

10^4
10^3
10^2
^{14}C-Thymine incorporation (c.p.m.)
30
60
120

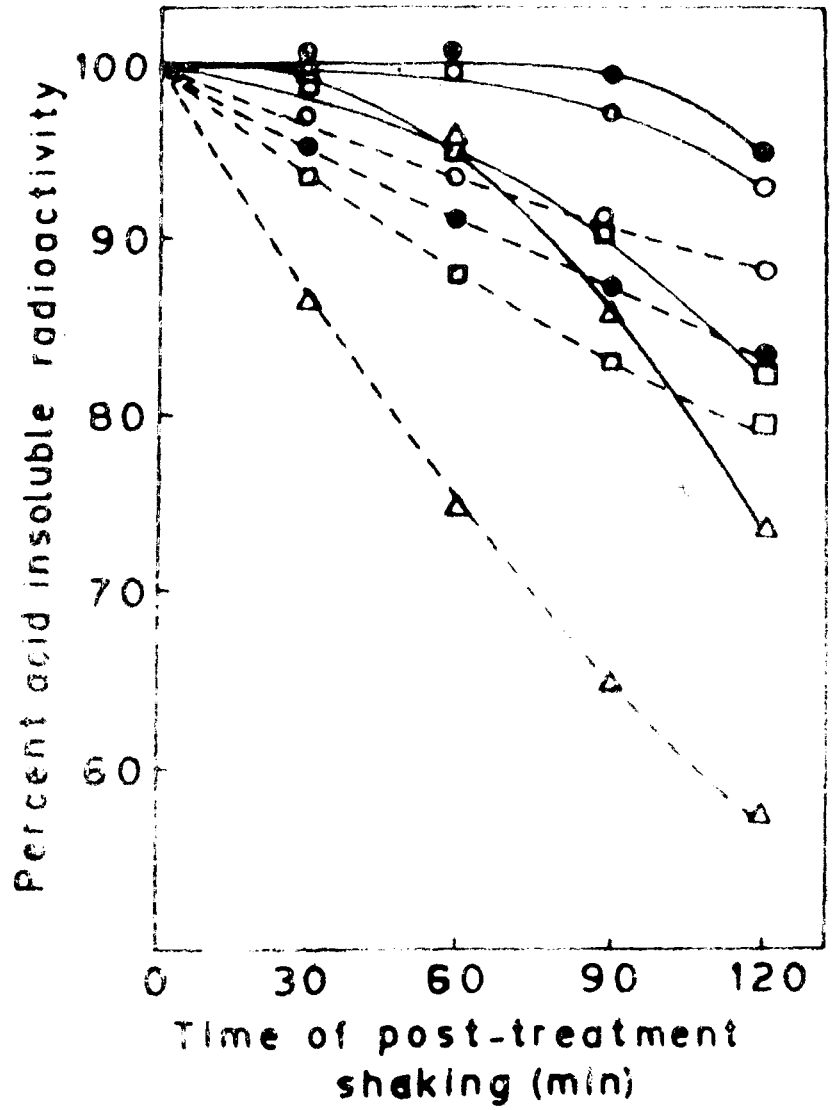
thymine exponentially and normal synthesis of DNA occurred. The heated wild-type (w.t.) bacteria exhibited an unexpected peak between 0 to 30 min and then there was a gradual increase in ^{14}C-thymine incorporation. As compared to unheated w.t. bacteria, incorporation of thymine by heated cells was less. During 2 hr of incubation, recovery of DNA synthesis was only 4-times compared to 30-times increase in colony forming ability. Unheated bacteria exhibited 12-times increase in ^{14}C-thymine counts under similar conditions. As compared to AB1157, AB2494 incorporated very little thymine. Poor incorporation compared well with the poor recovery of colony forming ability of AB2494 in recovery medium.

DNA degradation: The pattern of DNA degradation in unheated and heated bacteria is shown in Fig. 2. The w.t. and heat sensitive strains were tested for DNA degradation during liquid holding recovery. The initial radioactivity in the acid insoluble fractions of heated and unheated bacteria was less than 100% and varied from strain to strain, e.g. for unheated w.t., recA, lex and polA bacteria, the values were 95, 70, 80 and 75%. So far convenience, the initial counts were normalised to 100% and the rest of the results were calculated accordingly. DNA degradation was significantly inhibited in heated bacteria of all the strains.

Single-strand breaks and repair: To see that treatment to bacteria induces single strand breaks in bacterial DNA, bacteria were heated at 52°C for 15 and 30 min and the DNA

Fig. 2. DNA degradation during incubation in recovery medium at 37°C



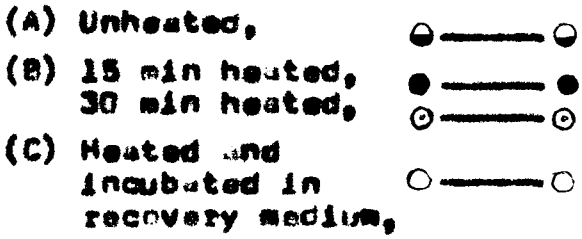

Percent acid insoluble radioactivity
100
90
80
70
60
0
30
60
90
120
Time of post-treatment shaking (min)

was analysed in alkaline sucrose gradients (Fig. 3). Compared to the sedimentation profile of unheated *E.coli* DNA, it was found that sedimentation profile of heated bacteria was different. The major peak shifted towards top of the gradient and the shift was proportional to the dose of heat. This suggested that low molecular weight DNA results due to single-strand breaks in DNA after heat-treatment. When 15 min heated bacteria were incubated in tryptone broth for 30 min and then analysed in alkaline sucrose gradients, DNA of normal molecular weight was found suggesting repair of single-strand breaks.

RNA synthesis: RNA synthesis was followed in unheated and heated AB1157 and AB2494. The incorporation of ^{3}H-uridine was measured (fig. 4). As observed with DNA synthesis, the incorporation of uridine was about 20-times in unheated bacteria after 2 hr of incubation. With heated cells, a peak was observed between 0 to 30 min followed by increase in incorporation of uridine which was about 11-times at the end of 2 hr of incubation. The incorporation of uridine by AB2494 was very poor as compared to the w.t. cells and unheated control.

RNA degradation: Degradation of RNA in unheated and heated w.t. and AB2494 strains is shown in Fig. 5. In unheated bacteria, both w.t. and lex, RNA degradation was not observed. With heated bacteria, there was a constant increase in ^{3}H-uridine counts released. The rate of RNA degradation in heated AB2494 was higher than that of w.t. bacteria. After 2 hrs of incubation

Fig. 3. Sedimentation patterns of ^{3}H-thymidine labelled DNA in alkaline sucrose gradients.



Vertical arrows indicate the position of ^{14}C-labelled λDNA used as internal reference in each gradient.

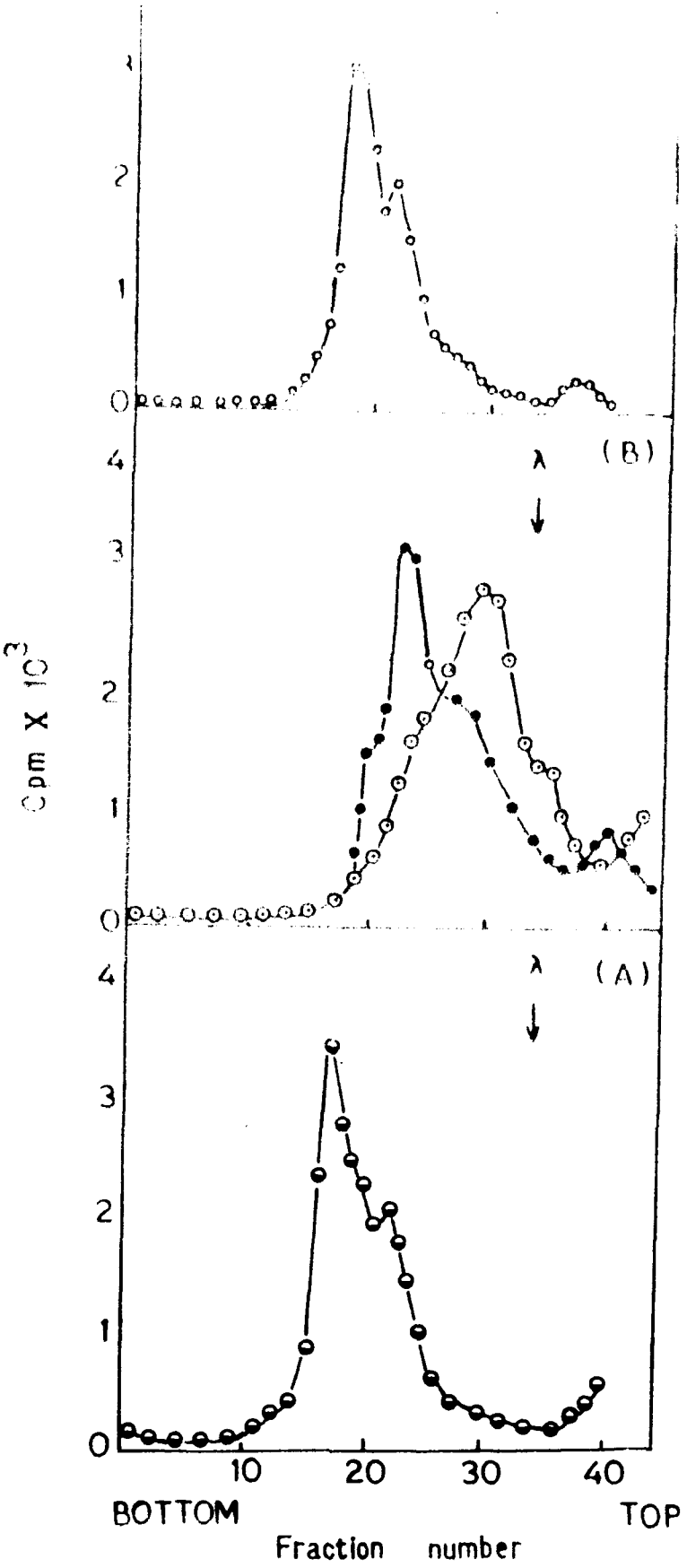
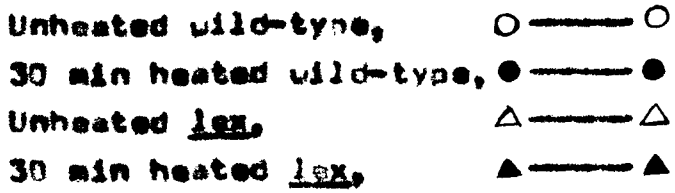

cpm X 10^3
(B)
(A)
λ
0
1
2
3
4
10
20
30
40
BOTTOM
TOP
Fraction number

Fig. 4. RNA synthesis patterns of wild-type and *lex* bacteria during incubation in recovery at 37°C



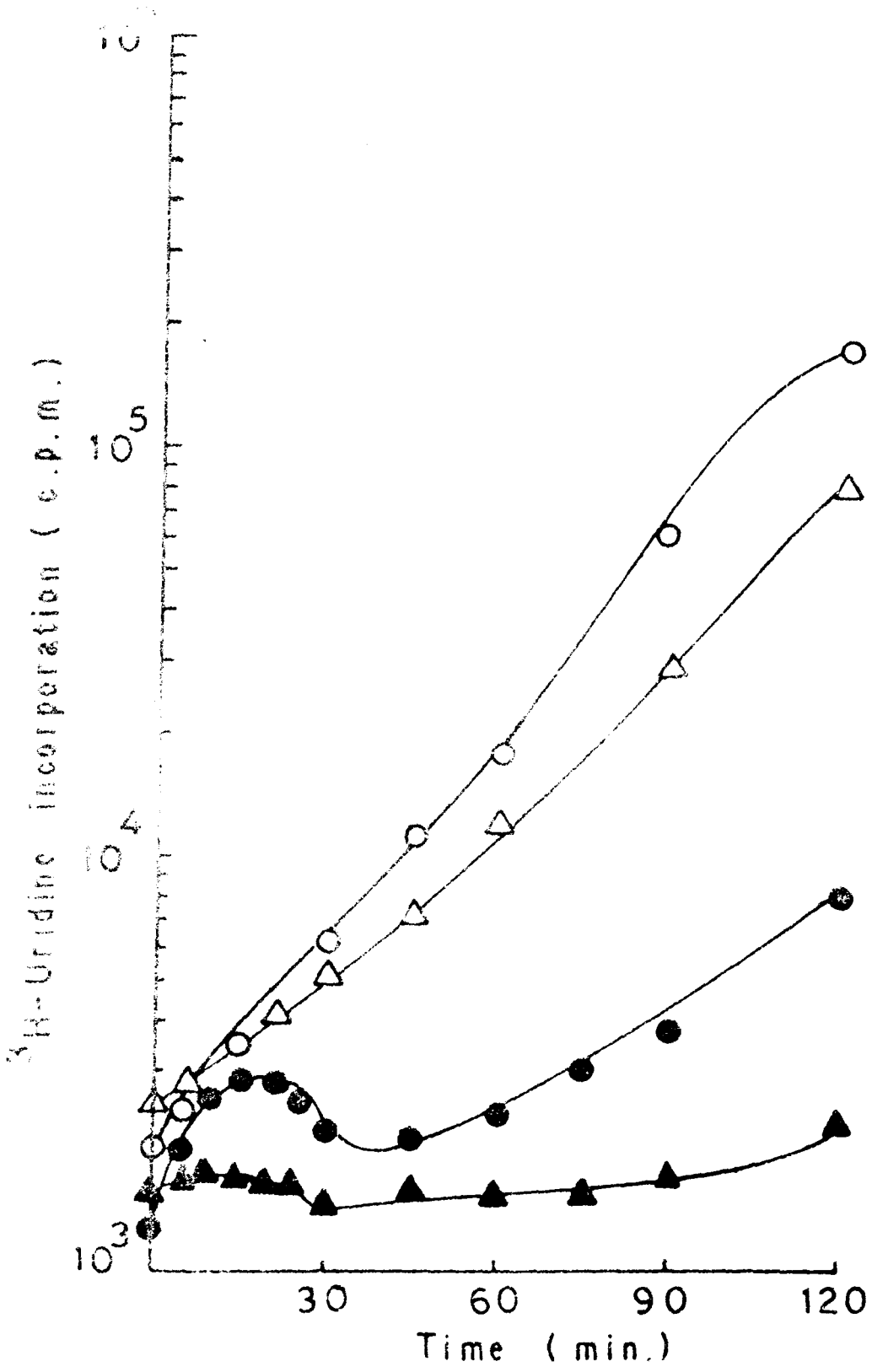
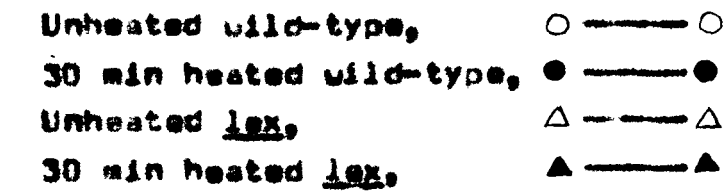
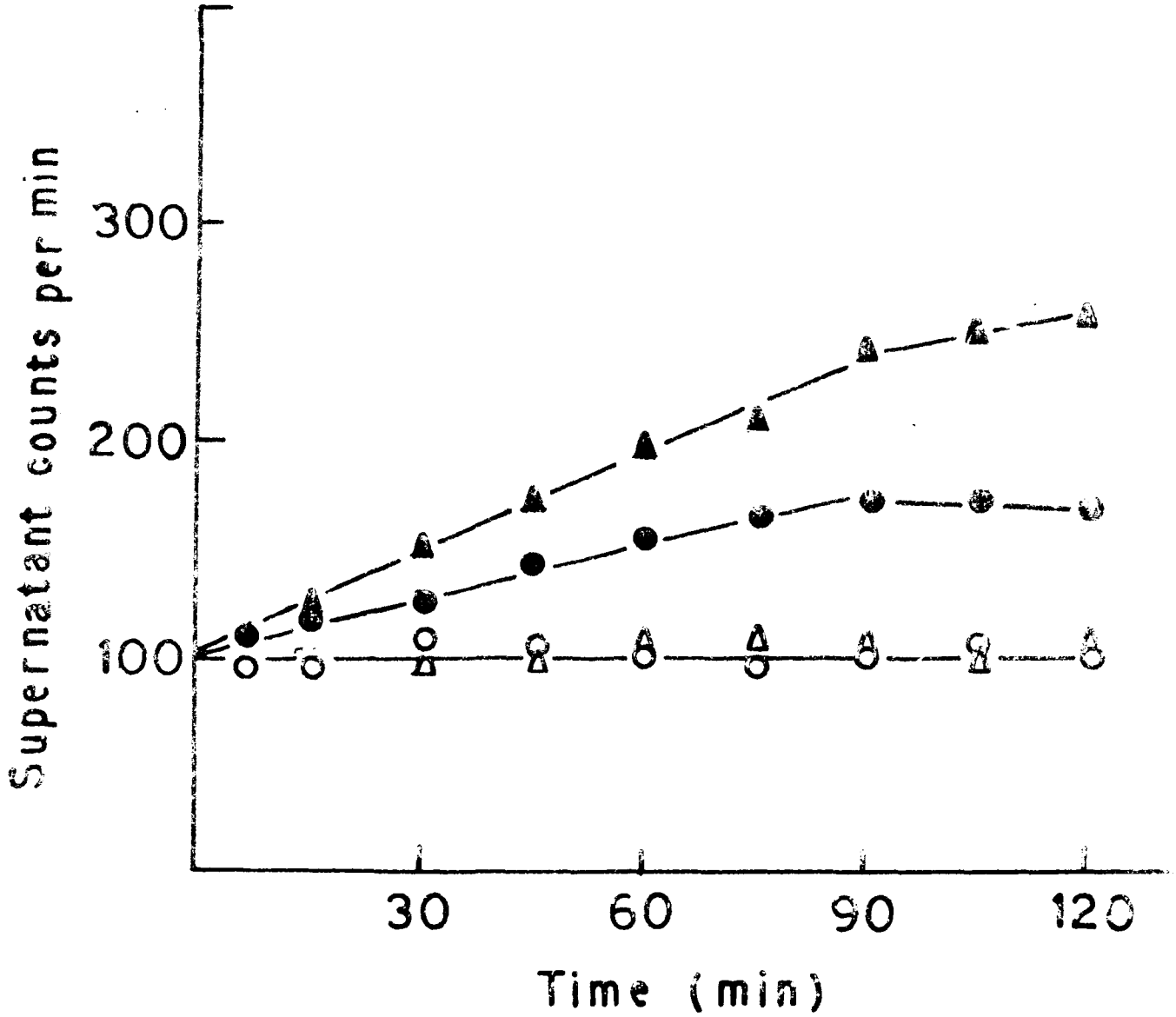

^{3}H-Uridine incorporation (c.p.m.)
10^5
10^4
10^3
30
60
90
120
Time (min.)

Fig. 5. RNA degradation patterns of wild-type and *lex* strains during the incubation in recovery medium at 37°C.




Supernatant counts per min
300
200
100
30
60
90
120
Time (min)

degradation was about 1.5 and 2.5-times compared to initial counts with AB1157 and AB2494 respectively. While RNA degradation stopped after 90 min in u.t. cells, it was observed upto 2 hr in AB2494.

Leakage of RNA: Leakage of RNA is shown in Fig. 6. During 30 min of heat treatment, u.t. and log strains released about 4% of their total RNA, out of which 3% leaked during 10 min of heat treatment.

Protein synthesis: Protein synthesis was measured in unheated and heated AB1157 and AB2494. The incorporation of ^{14}C-leucine was followed (Fig. 7). After 75 min of incubation, incorporation of leucine was observed with AB1.57 whereas there was no incorporation in AB2494 upto 2 hr. The heated cells exhibited an initial hump in incorporation.

Protein leakage: No leakage of protein was detected during heat treatment and during incubation at 37^{0}C in recovery medium. It was observed for both AB1157 and AB2494.

Permeability changes: As shown in Fig. 8, uptake of ^{3}H-actinomycin D increased with increase in heating. A 2.3 fold increase in uptake was found by 30 min heated AB1157 as compared to unheated bacteria. EDTA-treated cells incorporated more ^{3}H-activomycin D as compared to heated cells.

Although, the data has not been incorporated but it was observed that when heated bacteria was kept for 10 min in

Fig. 6. Permeability of wild-type bacteria during heat treatment.

^{3}H-activamycin D, Inward permeability to ○ ——— ○

^{3}H-uridine, Outward permeability to ● ——— ●

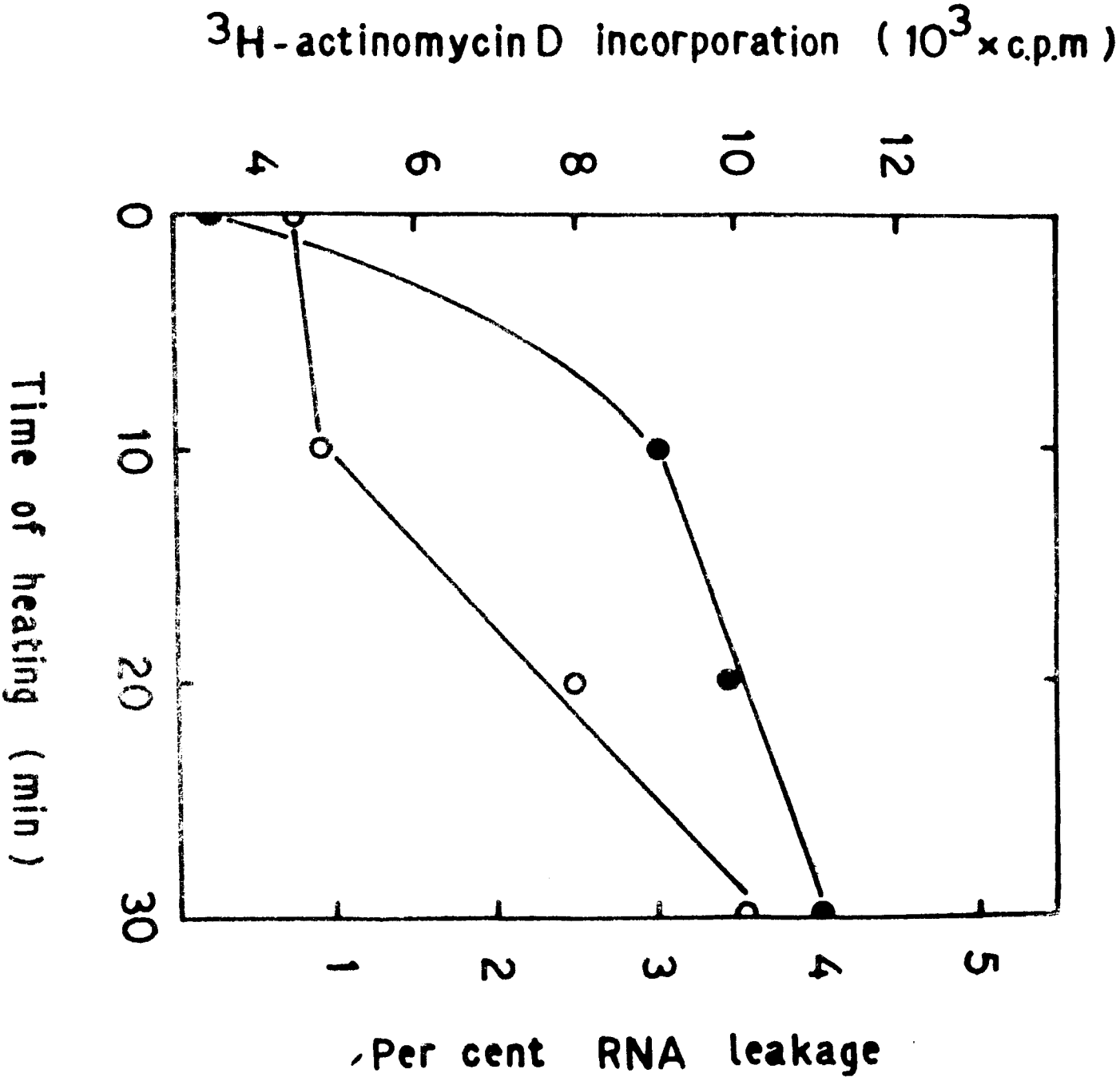
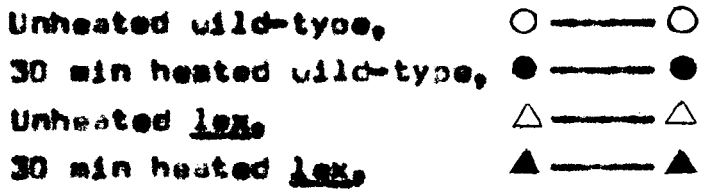
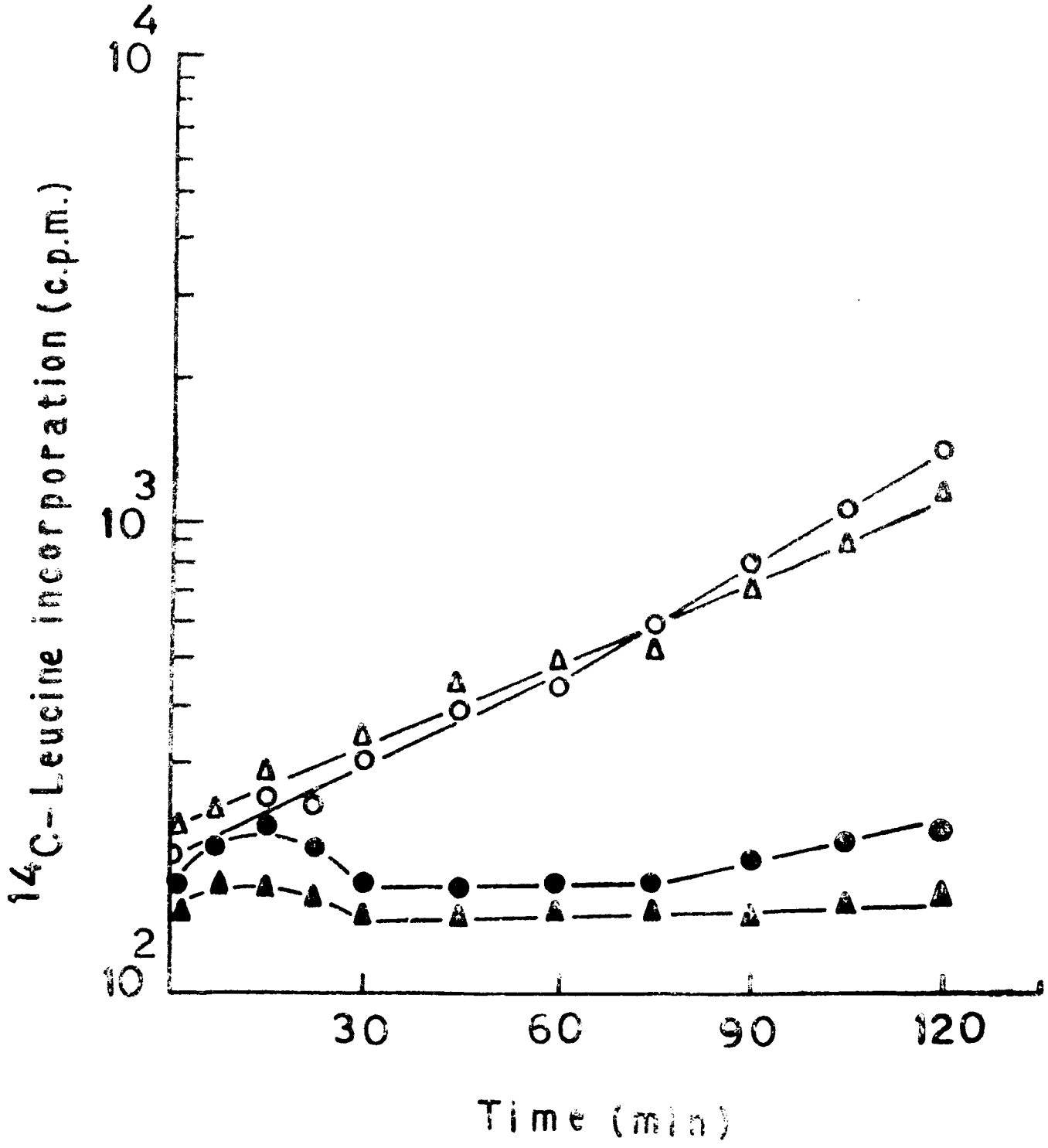

^{3}H-actinomycin D incorporation (10^3 x c.p.m)
4
6
8
10
12
0
10
20
30
Time of heating (min)
1
2
3
4
5
Per cent RNA leakage

Fig. 7. Protein synthesis in wild-type and *lex* bacteria.




10^4
10^3
10^2
^{14}C- Leucine incorporation (c.p.m.)
30
60
90
120
Time (min)

recovery medium an increased uptake of ^{3}H-actinomycin D was observed as compared to uptake of heated bacteria not held in recovery medium for 10 min. Further incubation in recovery medium showed less uptake. This pattern of increased uptake of ^{3}H-actinomycin D in 10 min was similar to the unusually higher uptake of ^{14}C-thymine, ^{3}H-uridine and ^{14}C-leucine during the first ten minutes (fig. 1,4,7). We explain this observation as hypertonic effect of the medium because heat treatment is always given to cells suspended in Tris-Mg buffer.

DISCUSSION

Heat treatment to bacteria caused inhibition of DNA synthesis. The degree of inhibition was more in *lex* strain than the wild-type strain. Even after 2 hr of liquid holding, there was insignificant increase in ^{14}C-thymine incorporation of *lex* bacteria. Since the w.t. and other bacterial strains recovered significantly during liquid holding in recovery medium and *lex* did not (Fig. 2d, Chapter IV), the results of DNA synthesis suggest that inhibition of DNA synthesis may be an important effect of heat, and *lex* may be required for resumption of DNA synthesis in heated bacteria. The effect of heat treatment on DNA synthesis of heated bacteria and state of synthesis in recovering bacteria have not been noted in earlier studies. However, Gomez *et al.* (1976) found that nalidixic acid treatment to heated cells did not interfere with recovery of viable counts.

Less DNA was degraded in heated bacteria compared to unheated corresponding strains. The inhibition of DNA degradation might be due to inhibition of DNase activity by heat. Chapman and Pollard (1969) reported inhibition of radiation induced DNA degradation when E.coli was heated at temperature greater than 45°C prior to γ-irradiation. This effect was attributed to the inhibition of DNases at higher temperature.

Single-strand breaks were observed in the DNA of heated bacteria. These breaks were repaired upon incubation of bacteria in tryptone broth for 30 min at 37°C. Single strand breaks (ssb) have been observed by other workers in the DNA of bacteria heated at 52°C which were repaired at 37°C (Bridges et al., 1969; Woodcock and Grigg, 1972). Breaks were not observed in DNA heated in vitro. Inman (1956) found that short periods of heat treatment caused local opening of DNA at various points. These specific regions are attacked by DNases (Goldmark and Linn, 1970). While ssb were induced in polA after exposure to 52°C, these were not found in the rec mutant of E.coli. It was,therefore, suggested that ssb are not produced by the direct action of heat-treatment (Sedgwick and Bridges, 1972).

Like DNA synthesis, synthesis of RNA remained inhibited for a short period in wild-type heated bacteria and then resumed exponentially. The synthesis of RNA in heated lex bacteria remained inhibited for longer period, thus suggesting the role of RNA synthesis in the recovery of heated

bacteria. In the previous chapter, we have shown that rifampicin, an inhibitor of de novo RNA synthesis, inhibited recovery of survival, and mitomycin C resistance of heated bacteria. Unheated bacteria increased 10-times in number during the 2 hr of liquid-holding recovery and incorporated 20-times ^{3}H-uridine at the end of 2 hr. On the other hand, heated bacteria increased only two times of the initial total counts while it incorporated 11-times ^{3}H-uridine at the end of 2 hr. This demonstrated that heated cells incorporated more ^{3}H-uridine than the unheated cells. These results suggested the involvement of de novo RNA synthesis in recovery of heated bacteria. As reviewed in the introduction of this chapter, the role of RNA synthesis in the recovery of several heated bacteria has been demonstrated.

RNA was degraded in heated cells during incubation in recovery medium. The extent of degradation was more in the lex mutant than the w.t. strain. This observation suggested that lex may be involved in the control of RNA degradation. It would be difficult to provide the mechanisms of regulation by lex^{+} but a model analogous to control of DNA repair and degradation proposed by Gudas and Pardee (1975) may be envisaged.

As a result of heating, RNA was degraded by enzymatic action of RNases. Heating also increases permeability of cells. Therefore, as a result of these two events, i.e. increase in permeability and enzymatic degradation of RNA, small pieces of degraded RNA leak out in the medium. We obtained maximum

leakage of RNA within 10 min of heating. Further, heating did not enhance leakage, although permeability of cells increased with increase in heat-treatment. The reason may be the inactivation of RNase(s) with increasing heat treatment.

Under similar conditions, leakage and degradation of protein was not detected. We have provided evidence that proteins are inactivated due to heat-treatment. The efficiency of repair enzymes involved in the repair of UV damage induced in DNA of E.coli was comparatively poor in heated bacteria. Liquid holding in recovery medium brought about resumption of their activity which was not inhibited by chloramphenicol. Similarly the enzymes involved in DNA degradation also lost their activity. The role of de novo protein synthesis in our experiments do not appear to play any major role.

The results on the uptake of ^{3}H-actinomycin C suggest that bacterial membrane was affected by heat which results in greater permeability of cell membrane. We observed leakage of RNA after heat-treatment and enhanced sensitivity to mitomycin C and actinomycin D.

CHAPTER VI

SURVIVAL OF BACTERIOPHAGE EXPOSED TO 52°C

INTRODUCTION

When Escherichia coli is exposed to 52°C, there occurs a loss in colony forming ability and the genes controlling sensitivity of E.coli to physical and chemical agents seem also responsible for sensitivity to heat treatment (Bridges et al., 1969; Ishi and Kondo, 1975; Chapter IV of this thesis). It has been suggested that common repair mechanisms act on DNA lesions induced either by ionizing radiation or by thermal treatment (Bridges et al., 1969). The repair of the lethal lesions induced in bacteria by heating to 52°C has been shown (Mukherjee and Bhattacharjee, 1970; Woodcock and Grigg, 1972; Chapter IV). Pollard and Soloska (1971) studied the inactivation of bacteriophages λ and T4 to thermal treatments higher than 60°C.

In this chapter, we have studied the effect of 52°C exposure on the plaque forming ability of bacteriophage λ. One of the purposes of this work is to check whether like E.coli, phage λ could be inactivated by 52°C and whether inactivation of λ is genetically controlled.

MATERIALS AND METHODS

Bacteria and phages: The following bacterial strains were used: AB1157, AB1886, AB2463, AB2470, AB2494, AB2480 and JG112.

The λ strains were λ $\underline{s}^+$, λ $\underline{cI}^{857}$, λ $\underline{h2}$, λ $\underline{cI}^{857}\underline{red1}$, λ $\underline{bio1}$ and λ $\underline{vir}$.

All have been described in chapter II.

Media, buffer and growth conditions: All the media and buffers have been described in chapter II. Over-night culture of bacteria was raised in tryptone broth. Fresh culture was raised in the same medium by diluting 50-times and growing upto $2-5 \times 10^8$/ml. TA7 was used for λ plating and the technique described by Adams (1959) was followed.

Preparation of phage lysates: Phage lysate were prepared in E.coli strain C600. Lysates were made on plates by confluent lysis. Bacteria from exponential culture, were harvested and suspended in Mg^{2+} solution. 0.3 ml of the bacteria was infected with phage λ. Phage λ was obtained from isolated plaques streaked on agar plates. Adsorption was allowed for 20 min at 37°C and plated with 3.0 ml of TA7. Plates were incubated at 37°C or 42°C (in case of λ $\underline{cI}^{857}$ phages) till confluent lysis was visible to naked eyes. The soft agar was scraped, 1% chloroform was added and after gentle vortexing, centrifugation was carried out in cold and the supernatant was collected. This is the phage lysate which was purified according to the technique developed in chapter III. Phage lysates were stored at 4°C.

Extracellular heat treatment to λ : Phage λ was suspended in Tris-Mg buffer. Approximately 10^8 phages/ml were subjected

to heat treatment in a waterbath maintained at 52°C (Chapter IV). Heated samples were withdrawn at regular intervals, suitably diluted and adsorbed to unheated bacteria at 37°C at low multiplicity of infection. After 20 min 3.0 ml TA7 was added and plated. After overnight incubation, plaque forming units (PFU) were counted.

Intracellular heat treatment of λ: Bacteria were harvested from exponential culture and suspended in Mg^{2+} solution. Phage λ was added at low multiplicity and adsorbed to bacteria at 37°C for 20 min. Finally, approximately 10^9 infective centres/ml ml were obtained and after removing unadsorbed phage, infected complexes were suspended in Tris–Mg buffer. Heat treatment was given at 52°C. Samples were withdrawn, suitably diluted and plated with 0.3 ml of the same unheated bacterial strain. PFU were counted after incubation of plates overnight at 37°C.

Adsorption and injection of λ to heated bacteria: In results of this chapter, we have followed the survival of unheated λ in heated host and a decline in PFU was observed. Therefore we checked if heated bacteria allowed poor adsorption and injection of unheated phage. Bacteria were heated upto 30 min. Unheated and heated bacteria were infected separately with unheated λ at low multiplicity at 37°C for 20 min. Unadsorbed phages were removed by two centrifugations in cold. Supernatants of the two samples were assayed for plaque forming units.

Phage λ was labelled with ^3H-thymidine. The methods of labelling and purification of phage are given in chapter V and

111 respectively. Injection of λ DNA was examined. Labelled phage was adsorbed to unheated and heated bacteria. Radioactivity in the pellet as well as supernatant was counted in Pakard Scintillation Counter.

Liquid-holding recovery: Phage λ heated intracellularly, was subjected to liquid holding recovery. Heated complexes were diluted 10-times on the recovery medium and incubated at 37°C for 1 hr. Samples were taken out at regular intervals to assay PFU.

52°C-induced g mutations of λg^+: λg^+ was exposed to 52°C as free particles or intracellularly for studying heat mutagenesis in λ. g mutants were scored according to the method described by Defais et al. (1971).

Pulse labelling with ^{3}H-uridine: The cells (2×10^8/ml) were treated for 30 min at 52°C. Unheated and heated cells were then diluted 10-times into recovery medium and incubated with shaking at 37°C. After suitable intervals, samples (1.0 ml) were taken out and added in tubes containing 5 μCi ^{3}H-uridine (specific activity, 10,600 mCi/mole) for 90 seconds. Cells were then fixed in chilled 2.0 ml TCA (10% w/v). After 30 min with TCA, the samples were filtered through millipore filter paper (Maxflow, India, pore size, 0.45 μ) and washed thoroughly with 2.5% chilled TCA solution. Acid insoluble fraction was counted in the liquid scintillation counter.

To measure mRNA synthesis in λ infected E.coli, infective centre were made as described above. Wild-type bacteria (2×10^8 cells/ml) were mixed together at high multiplicity of λ phage infection. 10mM KCN was added during adsorption of phage to bacteria to prevent phage multiplication. Unadsorbed phages were removed by centrifugation and washing with Tris-Mg buffer in cold. The infective centres were divided into two parts. One part was kept in cold, and the other was treated at 52°C for 30 min. After heat-treatment, KCN was removed from heated and unheated samples by centrifugation. Infective centres were diluted into rich medium containing 5 μCi/ml ^{3}H-uridine and 100 μg/ml adenosine. Further steps were the same as described for bacteria above.

Assay of bet-galactosidase:

(i) Construction of AB1157lac$^+$ from AB1157i-c$^-$ strain. Overnight culture of AB1157i-c$^-$ strain was prepared in (IXA) minimal medium supplemented with 0.5% glycerol and 40μg/ml of required growth factors. The same IXA medium containing 12 g/l agar-agar and 0.1% lactose was also poured on plates. All the three components were separately sterilized for 15 min at 15 lbs/sq inch and mixed at the time of pouring. 0.1 ml to 1.0 ml samples of overnight culture was spread on the plates and incubated at 37°C for 2 to 3 days. A few growing colonies appeared which were tested for sensitivity to λ and streaked on the minimal medium for isolated colonies.

(ii) Assay of beta-galactosidase activity. An over night culture of AB1157lac^+ was prepared at 37°C in IXA minimal medium supplemented with 40 µg/ml thiamin, $MgSO_4$ (0.001M), 40 µg/ml of amino acids required for growth and 0.5% glycerol. Bacteria were diluted 20-times in fresh medium and were allowed to grow for at least one doubling. Bacteria were chilled and then shaken at 37°C for 30 min (Miller, 1972). 40 tubes were taken with 0.5 ml of Z-buffer and one drop of toluene.

AB1157lac^+ was infected with λg90. Four flasks with 7.5 ml IXA medium supplemented with 1.5%(w/v) casamino acids was taken. Bacteria alone and phage-bacterial complexes were heated separately in Tris-Mg buffer. After heat-treatment, the cells were centrifuged, and the pellets were resuspended in fresh 7.5 ml medium, and incubated at 37°C in a metabolic shaker. At 0 min, 0.5 ml of bacteria and phage-bacterial complexes (both control and heated) were pipetted out and vortexed in tubes containing Z-buffer and toluene. After 20 min of incubation, 3.0 ml of 0.01M isopropyl-thio-beta-galactoside (IPTG) was added to the culture for induction of lactose operon. Samples were taken out at 5, 10, 15, 20, 30, 45, 60 and 90 min and vortexed in tubes containing the Z-buffer and toluene.

Toluene was allowed to evaporate by placing the tubes on a rotatory shaker at 37°C for 40 min with the tops open. The tubes were placed in a water-bath at 28°C for 5 min. The reaction was started by adding 0.2 ml of ortho-nitrophenyl-beta-D-galactoside (ONPG; 4 mg/ml). It was shaken for seconds. When sufficient yellow colour had developed, reaction was stopped after 15 minutes, by adding 0.5 ml of 1M Na_2CO_3.

β-galactosidase activity was determined by measuring the rate of change in optical density at 420 mμ and 550 mμ of a suspension of the toluene treated cells in reducing buffer containing ONPG. β-galactosidase activity is expressed in units of,

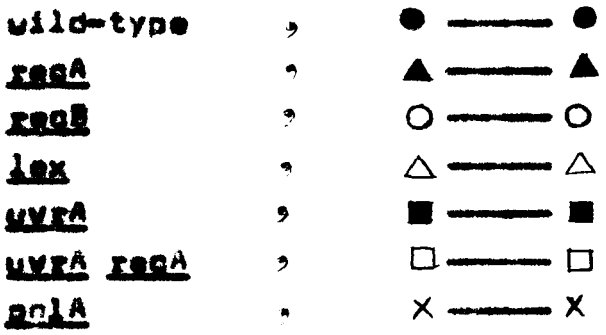
$(OD_{420} - OD_{550}/min/0.5$ ml of original culture) x 1000.

RESULTS

Survival of extracellularly heated λ : Extracellular heating of λg^+ at 52°C upto 60 min had no effect on plaque forming ability. Survival was identical on the wild-type (w.t.) and heat sensitive bacteria described in chapter IV. Similar results were obtained with λg1057, λb2, λvir and λbio1. Since there was no effect of heat on λ , the data are not shown.

Survival of intracellularly heated λ : Intracellular heating of phage λ resulted in loss of PFU. As compared to λ-w.t. complex, sensitivity of λ was increased in bacteria mutated in recA, lex, recB, uvrA and polA loci. λ was most sensitive in polA beats is to short exposures of heat (Fig. 1). λ rad was more sensitive to 52°C than λrad^+ phage, suggesting the role of rad^+ function in the repair of heat lesions (Fig. 2). A comparison of rad^+ and rad^- phages in recA, lex and uvrA strains revealed the additive effect of rad^+ with $recA^+$, lex^+

Fig. 1. Survival of intracellularly heated λ^+.



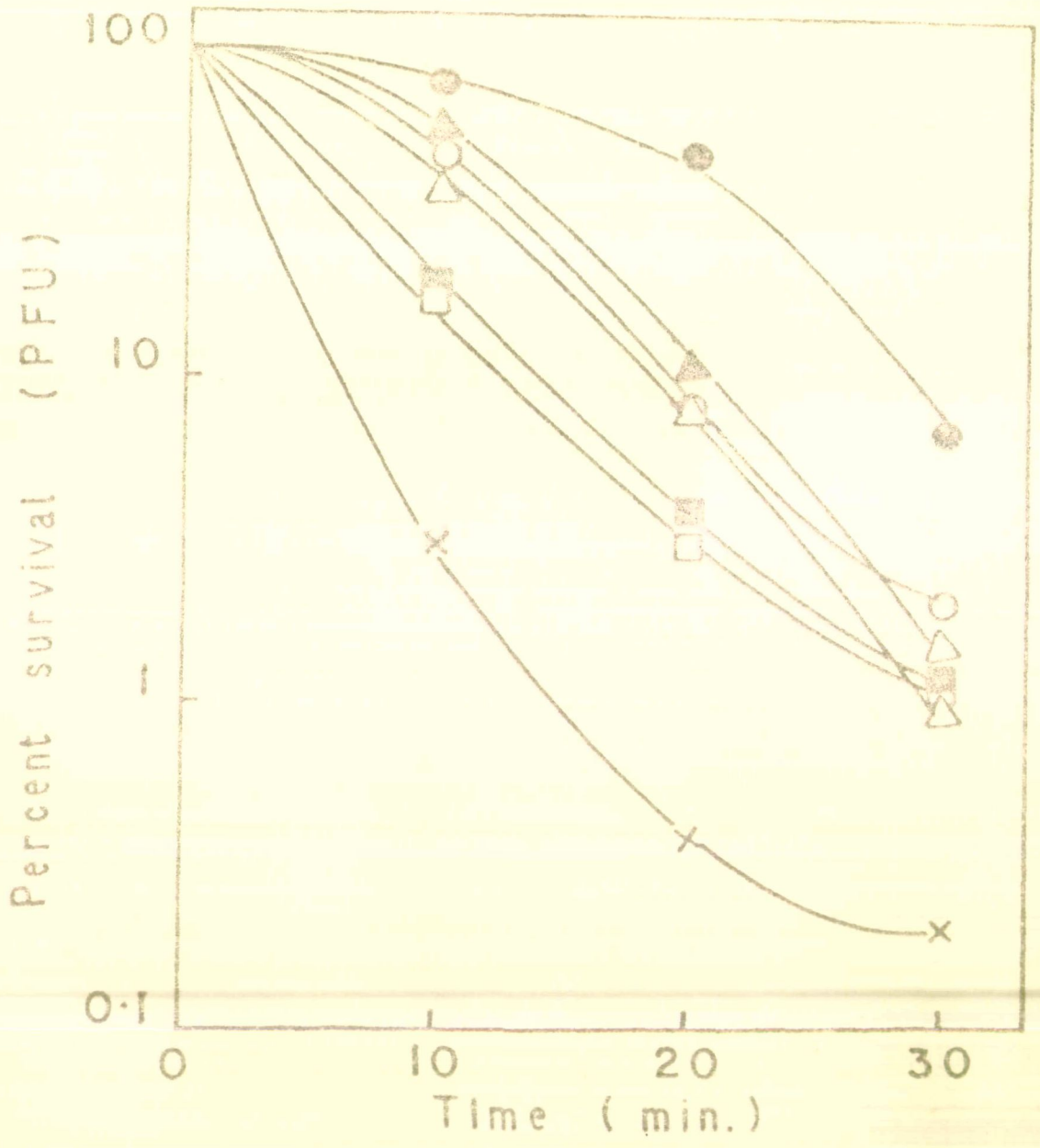
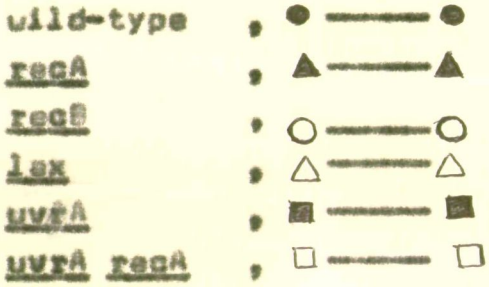
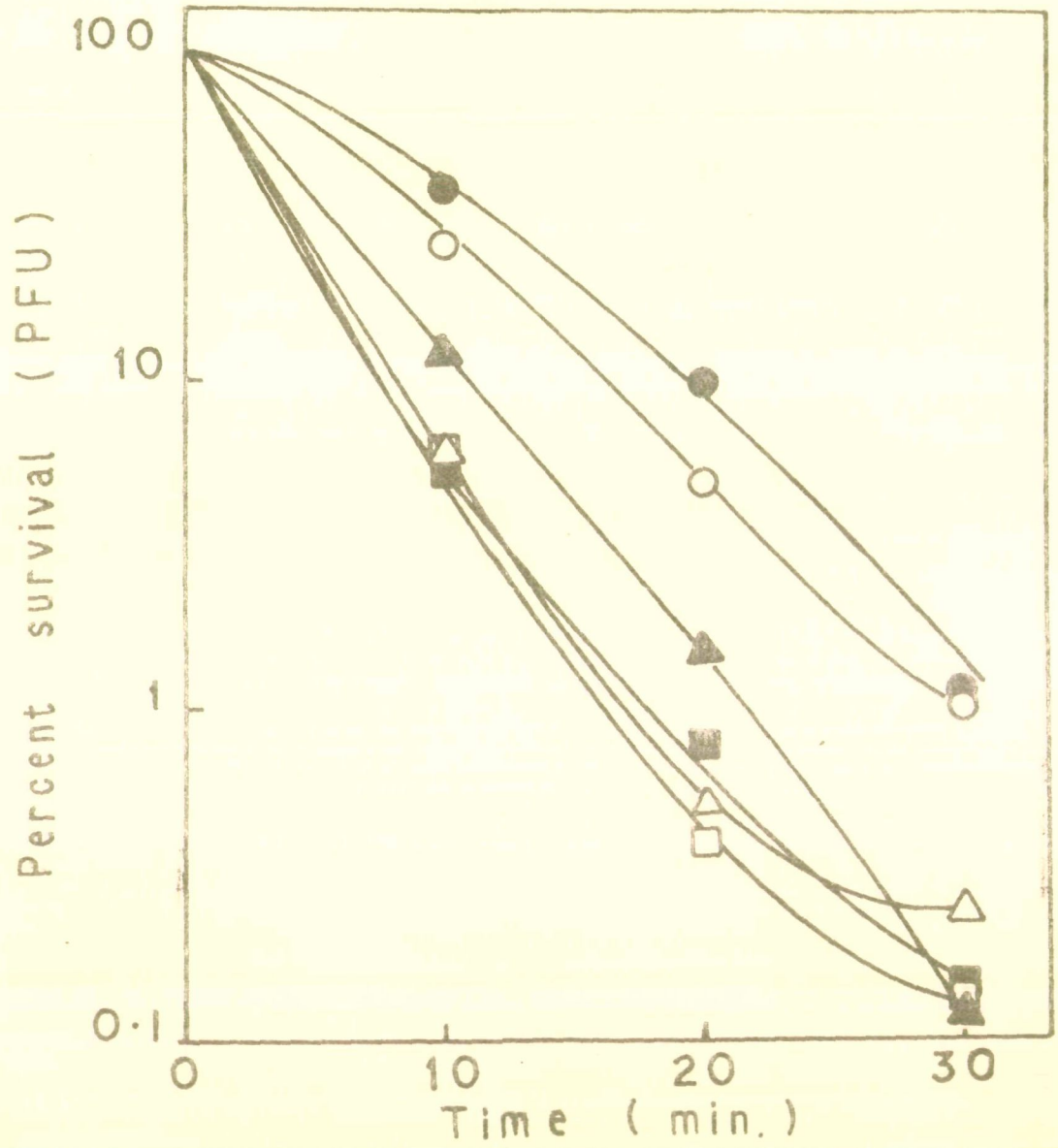

100
10
1
0·1
Percent survival (PFU)
0
10
20
30
Time (min.)

Fig. 2. Survival of intracellularly heated λ*red*.



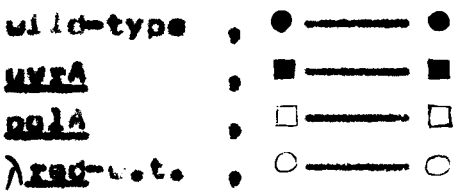

100
10
1
0·1
Percent survival (PFU)
0
10
20
30
Time (min.)

and *uvrA*$^+$ functions of *E.coli*. The shoulder of the curve observed at short exposures of heat with λ-*recA* complex was abolished with λ *red*-*recA* complex, Phage λ was sensitive in *uvrA* bacteria, and λ-*uvrA* and λ-*uvrA recA* showed identical sensitivity to heat.

Survival of unheated phage in heated bacteria: The survival of unheated phage in heated bacteria was followed (Fig. 3). Plaque forming ability of λ declined with heating of bacteria. However, as compared to the intracellular heating (Fig.1,2), the phages were less sensitive to heat (Fig.3). λ*red*$^-$ phage was more sensitive than the *red*$^+$ phage. We checked the possibility that inactivation of unheated λ in heated bacteria could be due to less adsorption and/or injection of λ. Adsorption and injection of λ was measured and were found to be normal (data is not shown).

Liquid holding recovery: When *E.coli* was heated and held at 37°C in tryptone broth, a significant recovery of colony forming ability was observed (Chapter IV). When λ-u.t. was held in recovery medium for 1 hr after heat treatment, no significant increase in PFU was observed (Table 1). There was no increase in PFU of λ-*recA* complex, however, λ-*polA* complex exhibited 3-times increase in PFU. Therefore, liquid holding recovery was not observed as with heated bacteria alone. We examined the possibility that the lack of recovery could be due to the inability of infested bacteria to recover in the recovery medium. Therefore, the activity of *lac* operon and

Fig. 3. Survival of unheated on heated *E.coli* K-12.



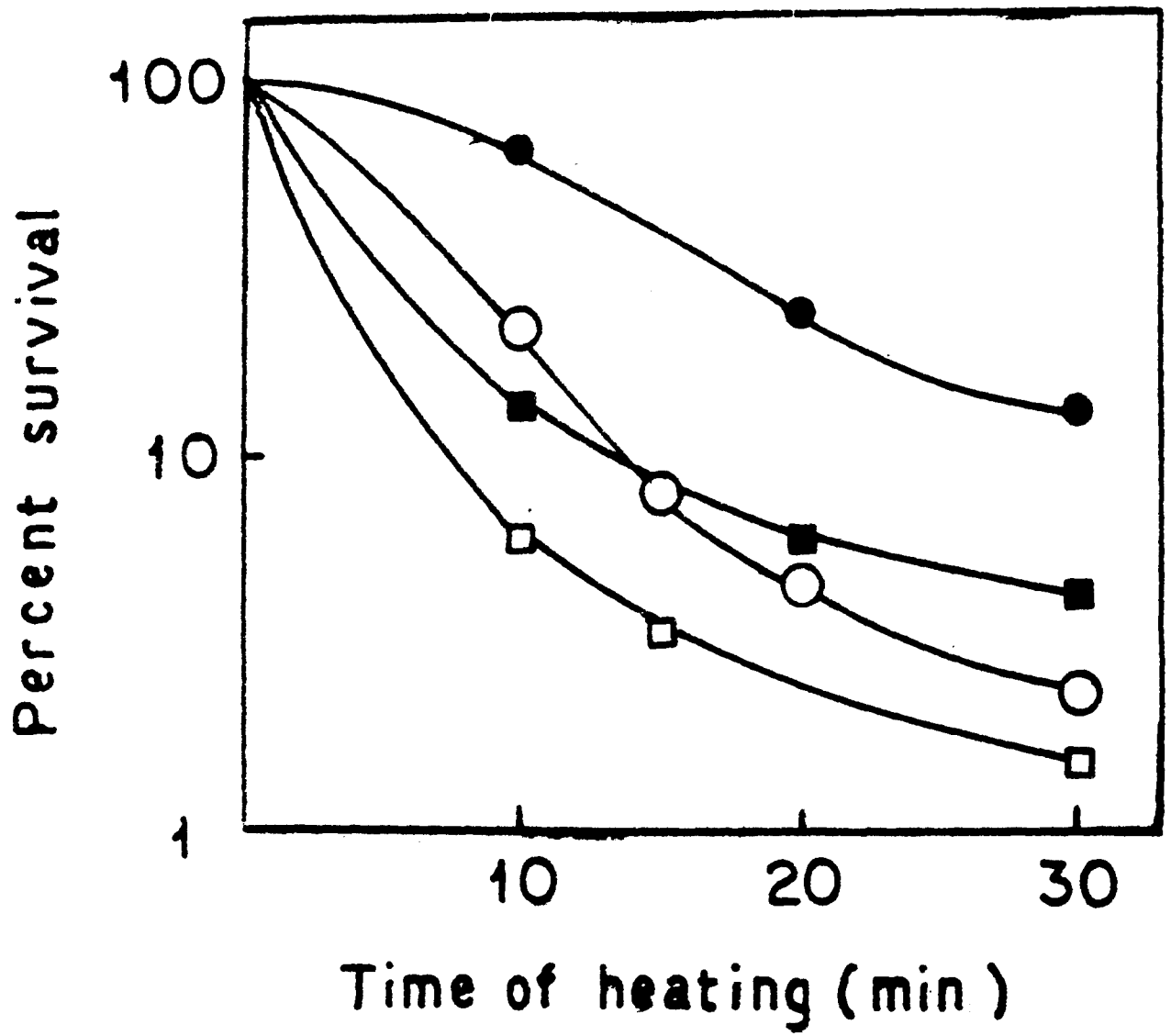

100
10
1
Percent survival
10
20
30
Time of heating (min)

Table 1: Liquid holding recovery of intracellularly heated phage λ.

λ-*E. coli* Complex	Fold-increase in the PFU during incubation in recovery medium at 37°C			
	Incubation for 30 min		Incubation for 60 min	
	Unheated complexes	Heated complexes	Unheated complexes	Heated complexes
λ-u.t.	30.0	1.0	201	1.0
λ-*recA*	26.0	1.1	85	1.4
λ-*lex*	22.4	1.0	90	1.0
λ-*rolA*	10.2	1.6	75	2.8

pulse-labelling of ^{3}H-uridine were examined in heated bacteria infected with phage λ.

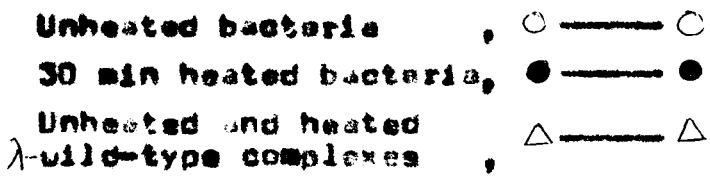
Activity of beta-galactosidase: Induction of β-galactosidase was normal in unheated bacteria AB1157 (Fig. 4). Heating of AB1157 for 30 min resulted in significant depression of β-galactosidase activity upto 60 to 90 min followed by the slow increase ~~increase~~ in enzyme activity. Enzyme activity was measured in unheated and 30 min heated λ-u.t. complexes. Enzyme activity remained inhibited in both unheated and heated complexes (Fig. 4).

mRNA synthesis: mRNA synthesis was measured in unheated and heated AB1157, and unheated and heated λ-u.t. complex by pulse-labelling with ^{3}H-uridine. With unheated bacteria alone incorporation of uridine followed exponentially (Fig. 5). Incorporation in heated bacteria was very less as compared to unheated bacteria. There was first a small peak and then a regular but poor incorporation of uridine was observed (Fig.5). The pattern was similar to the induction of β-galactosidase activity as shown above in Fig. 4.

With unheated λ-u.t. complex, a sharp increase in ^{3}H-uridine incorporation was observed which declined after 45 min. As compared to this, heated complex exhibited poor incorporation. A small peak was observed during early hours of incubation and a small rise at 90 min.

Heat-induced g mutants of λg^{+}: The data for induction of clear from turbid plaque morphology of λ is given in Table 2.

Fig. 4. Pattern of β-galactosidase activity in wild-type bacteria and λ-*E.coli* K-12 complexes.



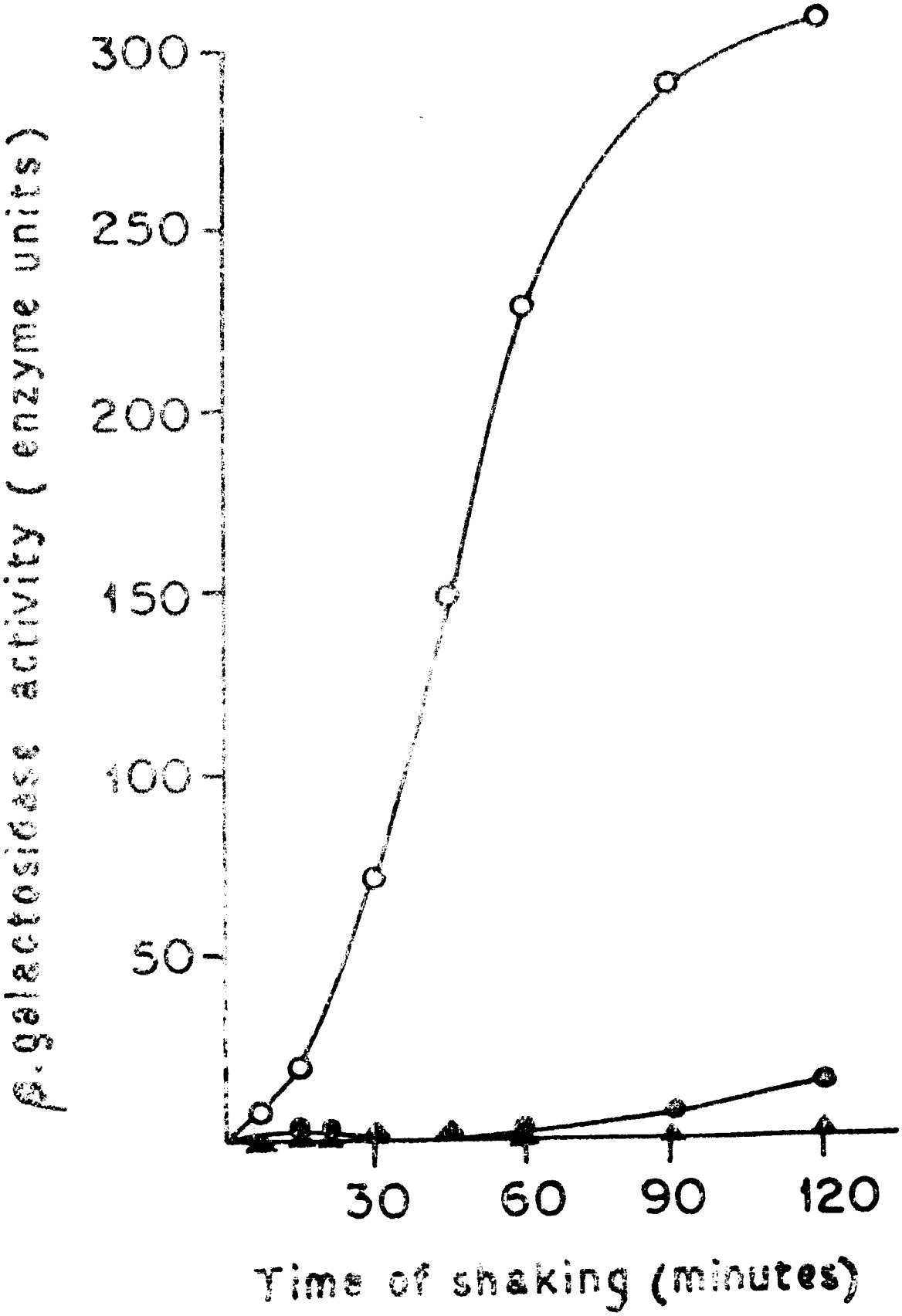
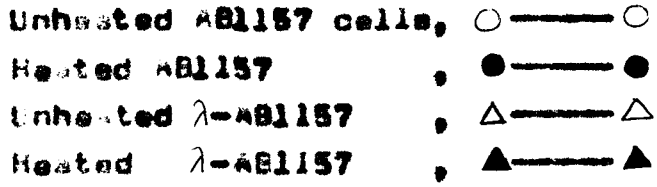

β-galactosidase activity (enzyme units)
300
250
200
150
100
50
30
60
90
120
Time of shaking (minutes)

Fig. 5. Pulse labelling experiment for mRNA synthesis.



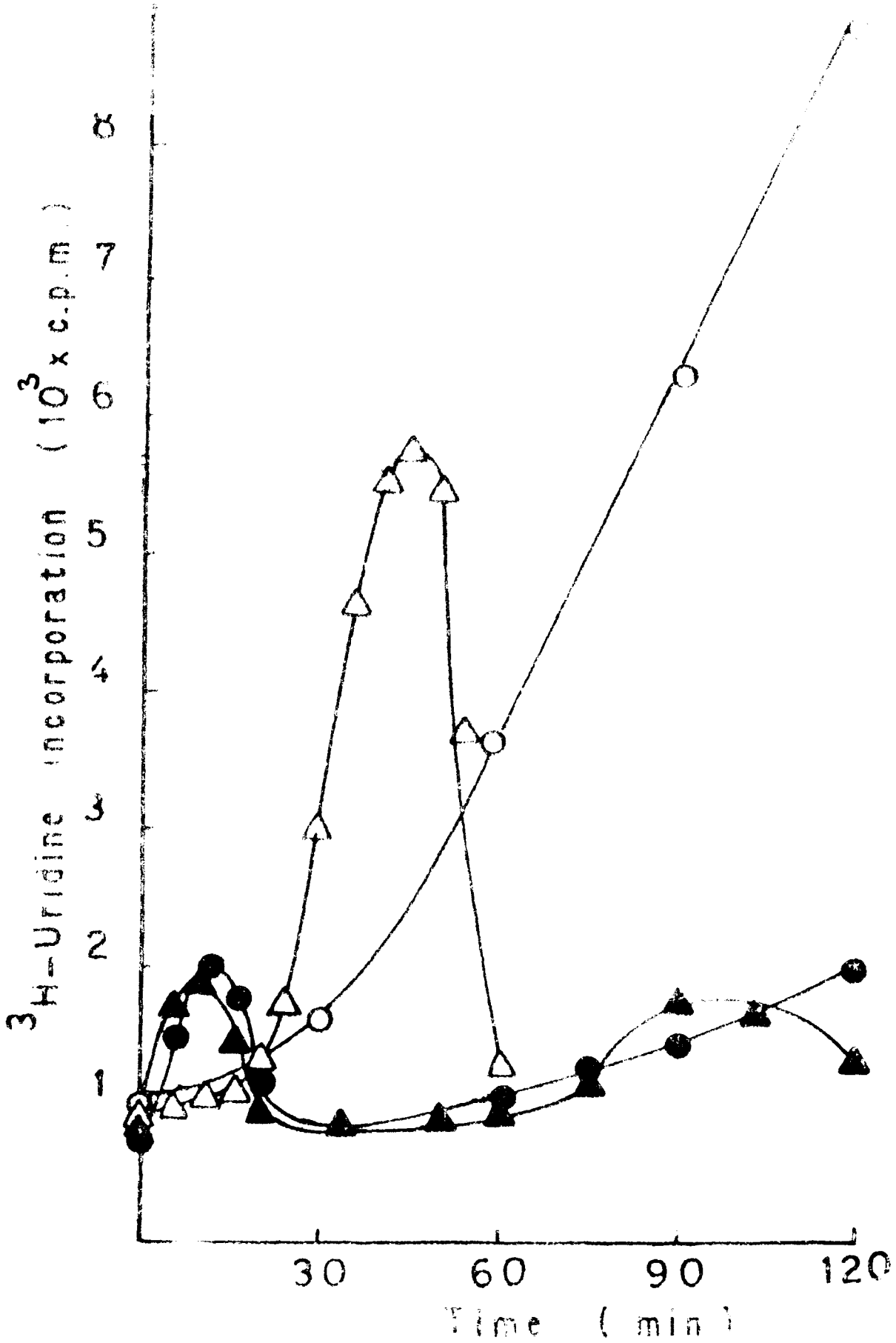

8
7
6
5
4
3
2
1
^{3}H-Uridine incorporation (10^3 x c.p.m.)
30
60
90
120
Time (min)

Table 2: Heat mutagenesis in phage λ .

Heat-treatment (min)	Mutation frequency per 10^3 PFU		
	Extracellularly heated λ	Intracellularly heated λ	Heating of u.t. bacteria alone
0	4.0	3.3	2.7
15	4.0	2.4	2.7
30	5.0	2.6	2.9

There was no increase in the mutation frequency of heated λ above the spontaneous level irrespective of whether the phage was heated extracellularly, intracellularly or infected to heated bacteria.

DISCUSSION

Extracellular heating of phage as free particles at 52°C had no effect on PFU. Even heating upto 60 min had no effect. This suggested that heat treatment did not alter adsorption of phage λ to *E.coli*, i.e. λ proteins were not affected. Nor it seems that phage DNA received any serious injury. Exposure of DNA to 52°C is known to cause transient local denaturation (Inman, 1966) which could be renatured in specific condition like slow cooling and low ionic strength (Marmur and Lane, 1960; Pollard and Sokesko, 1971). It may be possible that the heat lesions in DNA have been repaired due to slow post-treatment cooling at 37°C during adsorption and injection of λ to *E.coli*.

Intracellular heating of phage λ resulted in loss of PFU. λ was most sensitive in *polA* bacteria specially at short exposures of heat. The heat lesions might be single strand breaks (Bridges *et al.*, 1969; Woodcock and Grigg, 1972; Chapter V). The λ*red* phage was more sensitive than the *red*$^{+}$. This suggested that *red*$^{+}$ gene codes for a repair system involved

in the repair of heat lesions. The role of *rec*$^+$ in the repair of γ-rays-induced single-strand breaks has been demonstrated earlier (Srivastava, 1973). Since the shoulder of the curve obtained with λ-*recA* complex is abolished with λ *rec*-*recA*, it is suggested that *rec*$^+$ and *recA*$^+$ exert complementary effect specially at short exposures of heat.

Phage λ requires *uvrA*$^+$ gene for the repair of heat lesions when heated intracellularly. *uvrA* mutation had no marked effect on the survival of heated *E.coli* alone (Chapter IV). γ-rays irradiated phage λ also required *uvrA*$^+$ gene for some of the radiation induced lesions (Srivastava, 1973). Base modifications occur in phage T4 by heat treatment (Baltz *et al.*, 1976; Bingham *et al.*, 1976). Modified bases such as deoxyuracil is known to be removed from DNA by excision repair system (Lindahl, 1974; Friedberg *et al.*, 1975).

The observation that unheated λ was inactivated in heated bacteria suggested that multiplication of λ and its plaque forming ability depended on the host bacteria. The increased sensitivity of intracellular phages compared to sensitivity of unheated λ in heated hosts suggests that repair of DNA and its multiplication was influenced by the host which has also been heated.

As compared to *E.coli*, liquid holding recovery of PFU of λ was not observed when heated λ-host complex was held in recovery medium for 1 hr at 37°C. The lack of recovery may be

due to the inability of infected bacteria to recover in the recovery medium. As observed and concluded above, repair of DNA and its multiplication depended on the state of host. In the case of intracellular heating where the host has also been heated and injured, recovery involving de novo synthesis does not take place due to infection of phage. It is well known that DNA, RNA and protein syntheses of the host were inhibited following infection with λ (Cohen and Chang , 1970). There was no induction of β-galactosidase in infected bacteria irrespective of heat treatment. This might explain the lack of liquid holding recovery of intracellularly heated λ.

Therefore, it is concluded that extracellular heating of λ at 52°C had no effect on survival. Intracellular heating resulted in inactivation of PFU. The repair of heat lesions in DNA is controlled by *red*$^+$ gene of λ, and *rec*$^+$, *uvrA*$^+$, *polA*$^+$ and *lex*$^+$ genes of *E.coli*. Heating of bacteria alone has marked effect on phage survival. Heat treatment was not mutagenic to phage λ.

CHAPTER VII

GENERAL DISCUSSION AND CONCLUSION

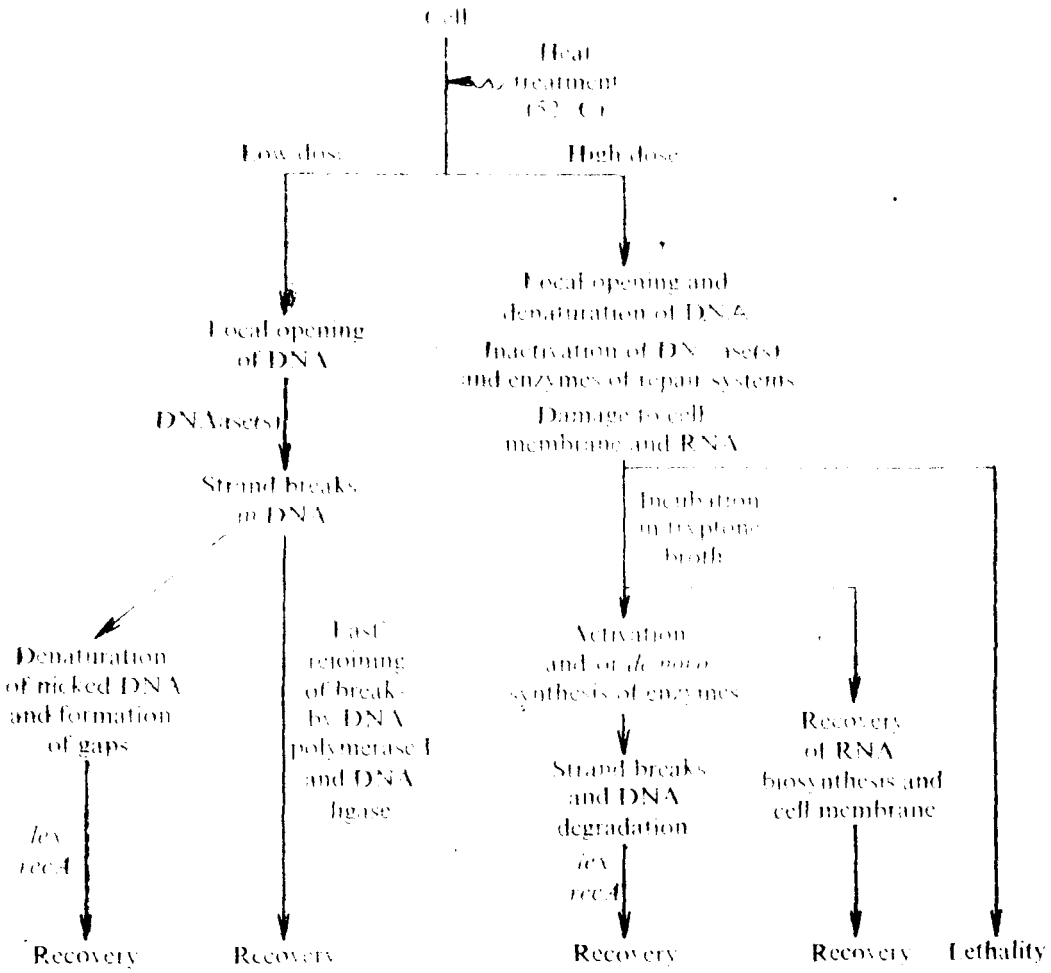
Some effects of heat treatment (52°C) to E.coli and bacteriophage λ was studied. In the case of bacteria, heat treatment caused loss of colony forming ability, and recA, lex and polA strains were more sensitive than the wild-type, recB and uvrA strains. Liquid holding recovery was influenced by lex function of E.coli. Heat treatment affected metabolism and stability of DNA and RNA and caused damage to cell membrane and enzyme. Phage λ , on the other hand, was not inactivated at 52°C as free particles. However, intracellular heating inactivated λ and the sensitivity depended largely on bacterial strain.

On the basis of these data and those reported earlier (as reviewed in Chapters IV and V), a scheme for heat lesions and their mode of recovery is being proposed (Fig. 1).

Short periods of heat treatment (low doses) cause transient local openings of DNA at various points (Inman, 1966). *In vivo*, these specific regions are recognised and attacked by DNase(s) resulting in nicked DNA (Goldmark and Linn, 1970; Sedgwick and Bridges, 1972). As the heat treatment prolongs, nicked ends get denatured and DNase(s) also become inactive.

The local openings induced in λDNA by extracellular heat treatment are renatured due to slow cooling during infection. However, if λDNA was heated intracellularly in the host, nuclease(s) induce single strand breaks followed by formation of gaps which would require the activity of repair enzymes. It has been proposed earlier that single-strand

Fig. 1. Scheme of *in vivo* heat (52°C) induced lesions and their mode of repair in *Escherichia coli* K-12.


Cell
Heat treatment (52 °C)
Low dose
High dose
Local opening of DNA
DNA(ases)
Strand breaks in DNA
Denaturation of nicked DNA and formation of gaps
lex recA
Recovery
Fast rejoining of breaks by DNA polymerase I and DNA ligase
Recovery
Local opening and denaturation of DNA
Inactivation of DN ase(s) and enzymes of repair systems
Damage to cell membrane and RNA
Incubation in tryptone broth
Activation and de novo synthesis of enzymes
Strand breaks and DNA degradation
lex recA
Recovery
Recovery of RNA biosynthesis and cell membrane
Recovery
Lethality

breaks are not formed as a direct consequence of heat but breaks appear as a result of nuclease action (Sedgwick and Bridges, 1972). Nicked DNA will be subjected to fast rejoining by DNA polymerase I and ligase (Town et al., 1971, 1972; Srivastava, 1974), while the remaining breaks/gaps require functional lex and recA genes.

For the repair of λ DNA, the gene-products of polA$^+$, uvrA$^+$, lex$^+$ and rec$^+$ of E.coli and red$^+$ of λ are required. The uvrA$^+$ gene plays an important role in the repair of λ DNA rather than in the repair of bacterial DNA. Thus, it seems that at low doses only the lesions in the DNA are important and these lesions are repairable (Woodcock and Grigg, 1972; Chapter VI).

Contrary to radiation, heat induces multi-target damage including DNA, RNA, protein and cell membrane. The damages other than DNA are more important at longer periods of heating (high dose). Damage to DNA would involve massive denaturation of DNA upto a certain point when nuclease(s) and other enzymes involved are inactivated by heat. DNA synthesis is inhibited and normal synthesis resumes during incubation in recovery medium.

RNA synthesis is susceptible to heat and is probably due to the attack by RNase(s) which become inactivated at higher doses of heat. Liquid holding requires de novo RNA biosynthesis. It is a hypothesis that lex$^+$ gene plays a role in the control of RNA degradation after heat treatment.

Intracellularly heated λ cannot recover during liquid holding in recovery medium, most probably because of the inhibition of de novo host biosyntheses following phage λ infection which are vital for repair and multiplication of λ.

Inactivation of repair enzymes at longer periods of heat treatment constitutes an important effect of heat. We have demonstrated inhibition of DNA degradation and inactivation of repair enzymes. All other proteins including nucleases, proteinases and enzymes of transport system may be inactivated by heat. Most of these proteins and enzymes are renatured/ activated without involving de novo protein synthesis during incubation in recovery medium. For example, chloramphenicol had no effect on the recovery of normal UV resistance of heated bacteria.

Heat treatment also affects the bacterial cell wall and damages the permeability barrier, as a result permeability increases and cells become less tolerant to osmotic shock. Recovery of the cell membrane occurs in recovery medium as shown by high salt tolerance and decreased uptake of ^{3}H-actinomycin D.

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Effect of Incubation Media on the Recovery of *Escherichia coli* K12 Heated at 52 °C

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The exposure of exponentially grown *Escherichia coli* K12 to 52 °C for 30 min in Tris/Mg^{2+} buffer resulted in a considerable loss of viability when plated on tryptone agar. When such heated bacteria were held at 37 °C for 2 h in tryptone broth before plating on tryptone agar, there was a significant increase in viability. Thus, heat damage was repaired in tryptone broth but not on tryptone agar. Recovery was greater in tryptone broth than in synthetic medium. In tryptone broth, *recA* or *polA* mutants also recovered but a *lex* mutant did not.

As a result of heating, the sensitivity of bacteria to ultraviolet radiation (u.v.), to mitomycin C and to plating on high salt medium was enhanced. After incubation for 2 h in tryptone broth at 37 °C, the bacteria regained their resistance to u.v. and mitomycin C and tolerance to high salt medium. Recovery of viability required RNA and protein synthesis, whereas recovery of u.v. resistance did not require protein synthesis. Heating for 30 min inhibited the release of acid-soluble material from DNA in all strains of *E. coli* used.

INTRODUCTION

Injury to bacteria caused by exposure to high temperatures and their subsequent recovery has been studied in *Staphylococcus aureus*, *Streptococcus faecalis*, *Bacillus subtilis*, *Salmonella typhimurium* and *Pseudomonas fluorescens* (Iandolo & Ordal, 1966; Clark, Witter & Ordal, 1968; Bluhm & Ordal, 1969; Rosenthol & Iandolo, 1970; Tomlins & Ordal, 1971; Miller & Ordal, 1972; Gray, Witter & Ordal, 1973). The heat injuries have been characterized as damage to the cell membrane, degradation of ribosomal RNA and alteration in enzyme activity. Heat treatment of *Escherichia coli* at 52 °C apparently induces single-strand breaks in the DNA and post-treatment incubation in phosphate buffer leads to the rejoining of these breaks and an increase in colony-forming ability (Bridges, Ashwood-Smith & Munson, 1969; Mukherjee & Bhattacharjee, 1970; Woodcock & Grigg, 1972). Bridges *et al.* (1969) demonstrated that mutations in certain loci, which render *E. coli* sensitive to radiation, also enhance its sensitivity to heat. The work reported here was undertaken in an attempt to understand the nature of heat lesions in *E. coli* K12, the requirements for recovery and the involvement of known genetic loci. Recovery is defined as an increase in the viability of heated bacteria by incubation in recovery medium before plating on tryptone agar, compared with immediate plating on tryptone agar.

METHODS

Bacteria. These are listed in Table 1.

Media. The minimal glucose salts medium (MM) contained (per litre): $K_2HPO_4.3H_2O$, 7 g; KH_2PO_4, 3 g; $(NH_4)_2SO_4$, 1 g; $MgSO_4.7H_2O$, 0·1 g; sodium citrate.$2H_2O$, 0·5 g; $MnSO_4$ and $Fe_2(SO_4)_3$, each 0·1 ml of 0·01 M solutions; and thiamin, 1 mg. D-Glucose [50 ml of a 10% (w/v) solution] was sterilized separately

T-13

Table 1. *Escherichia coli* K12 strains used

Strain	Relevant genetic markers	Source
AB1157	*thi argB thr leu pro his*	P. Howard-Flanders
AB2463	*recA13 thi argB thr leu pro his*	P. Howard-Flanders
AB2470	*recB21 thi argB thr leu pro his*	P. Howard-Flanders
AB1886	*uvrA6 thi argB thr leu pro his*	P. Howard-Flanders
AB2494	*lex thi thr leu pro his metB*	P. Howard-Flanders
JG112	*polA1 thi thyA*	J. Gross

and then mixed with the salts. The medium was supplemented with required amino acids at 40 μg ml^{-1}. The thymine requirement of *E. coli* JG112 was 20 μg ml^{-1}. Tryptone broth (RM) contained (per litre): tryptone (Oxoid), 10 g; yeast extract paste (Centron Research Laboratories, Pvt. Ltd, India), 5 g; and NaCl, 5 g. Bacteria were always plated on tryptone broth containing 1·5% (w/v) agar (BDH). The high salt medium consisted of tryptone agar containing 2% (w/v) NaCl, on which *E. coli* grows with no detectable loss of viability.

Buffers. All dilutions were made in 0·01 M-$MgSO_4$ solution. Bacteria were heat-treated in Tris/Mg^{2+} buffer (0·01 M, pH 8·0).

Heat treatment. Washed bacteria from an exponentially growing culture (1×10^8 to 5×10^8 viable units ml^{-1}) were suspended in buffer in a test tube and placed in a water bath at 52 °C. The temperature in the tube rose to within 0·5 °C of the final temperature in 1 min. After heat treatment, samples were diluted and immediately plated to assay colony-forming ability.

Post-treatment incubation. Heated and unheated bacterial suspensions (0·5 ml) were diluted 10-fold into MM or RM and shaken for 2 h at 37 °C. At intervals, samples were diluted and plated. Where stated, chloramphenicol (Cam; 100 μg ml^{-1}) or rifampicin (Rif; 10 μg ml^{-1}) was added to the recovery medium (these were gifts from May & Baker, India, and Professor G. C. Lancini, respectively). Before plating, the bacteria were washed to remove the drugs. Neither antibiotic reduced the viability of unheated bacteria at the concentrations used.

Exposure to ultraviolet radiation (u.v.) and mitomycin C. Unheated bacteria, heated bacteria and heated bacteria held in recovery medium for 2 h with and without chloramphenicol were compared for u.v. and mitomycin C sensitivity. To measure u.v. sensitivity, bacteria were plated on tryptone agar and the plates were exposed to 254 nm and incubated at 37 °C. Irradiation and incubation were performed in complete darkness to avoid photoreactivation. Sensitivity to mitomycin C (Calbiochem) was determined by plating the bacteria on tryptone agar containing the drug at 0·5 μg ml^{-1}. Mitomycin C at this concentration had no effect on the viability of unheated bacteria.

DNA degradation. Bacteria were radioactively labelled by growing them in minimal medium supplemented with their required growth factors and 5 μCi [^{3}H]thymidine ml^{-1} (sp. act. 6500 mCi mmol^{-1}; Bhabha Atomic Research Centre, India). Labelled bacteria were heated for 30 min at 52 °C and incubated with shaking in RM. Samples (0·2 ml) were removed at regular intervals, fixed for 30 min in 1 ml chilled 5% (w/v) trichloroacetic acid and the acid-insoluble fractions were collected on Millipore filters (pore size, 0·45 μm). The radioactivity was measured in a liquid scintillation counter.

RESULTS

Survival of heated bacteria

The survival of bacteria when plated directly on to tryptone agar immediately after heating is shown in Fig. 1. The mutants *recA*, *lex* and *polA* were more sensitive to heat than the wild type. Mutants *uvrA* and *recB* were as resistant as the parent strain (data not shown). The wild type and the *recA* mutant were more sensitive when grown in MM than when grown in RM. The viability of *polA*, and to a large extent *lex*, was not affected by the growth medium.

Liquid holding recovery in RM and MM

The bacteria were heated for 30 min at 52 °C and then shaken for 2 h in MM or RM. Colony-forming ability was assayed at intervals during this period by plating samples on tryptone agar. The viability of unheated bacteria under the same conditions was also assayed (Fig. 2). Recovery was influenced by the composition of both the pre- and post-

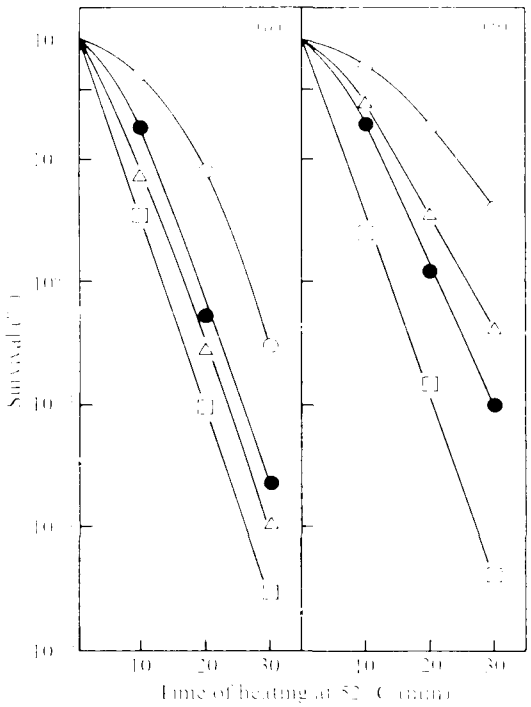

Fig. 1. Survival of bacteria heated at 52 °C after growth in (*a*) MM and (*b*) RM: ○, wild type; ●, *lex*; □, *recA*; ■, *polA*.

treatment media. Recovery is the ratio of the viable numbers of heated and unheated bacteria at 2 h.

The results shown in Fig. 2(*a*, *c*) are for bacteria grown in MM and RM, respectively, and shaken in MM after heat treatment. When grown in MM before heat treatment, the wild type and *recA* strains showed recovery during the 2 h incubation, whereas *lex* and *polA* mutants did not (Fig. 2*a*). When grown in RM before treatment, the colony-forming ability of *recA* and *lex* declined, whereas with the wild type loss of viability occurred for 1 h (Fig. 2*c*): again, *polA* cells did not recover.

The results shown in Fig. 2(*b*, *d*) are for bacteria grown in MM and RM, respectively, and shaken in RM after heat treatment. Except for the *lex* mutant, all the strains recovered significantly. The apparent extent of recovery after 2 h for the wild type, *recA*, *polA* and *lex* strains was 3, 7, 10 and 1, respectively (Fig. 2*d*). However, the *lex* mutant failed to recover and the increase in viable counts of the *lex* mutant was probably due to the multiplication of survivors since under identical conditions the increase in the number of unheated bacteria was the same.

When bacteria grown and treated as in Fig. 2(*d*) were not shaken but simply held in tryptone broth, the same results were obtained. Thus, holding in tryptone broth for 2 h after heat treatment increased survival compared with immediate plating on tryptone agar. Tryptone broth can therefore be referred to as recovery medium.

U.v. and mitomycin C sensitivity and salt tolerance

The parent strain, AB1157, was heated for 30 min. Half the culture was exposed to u.v., mitomycin C or plated on high salt medium and the colony-forming units were counted. The other half was suspended in recovery medium for 2 h and then exposed to u.v., mitomycin C or high salt medium. Parallel controls of unheated bacteria were also carried out.

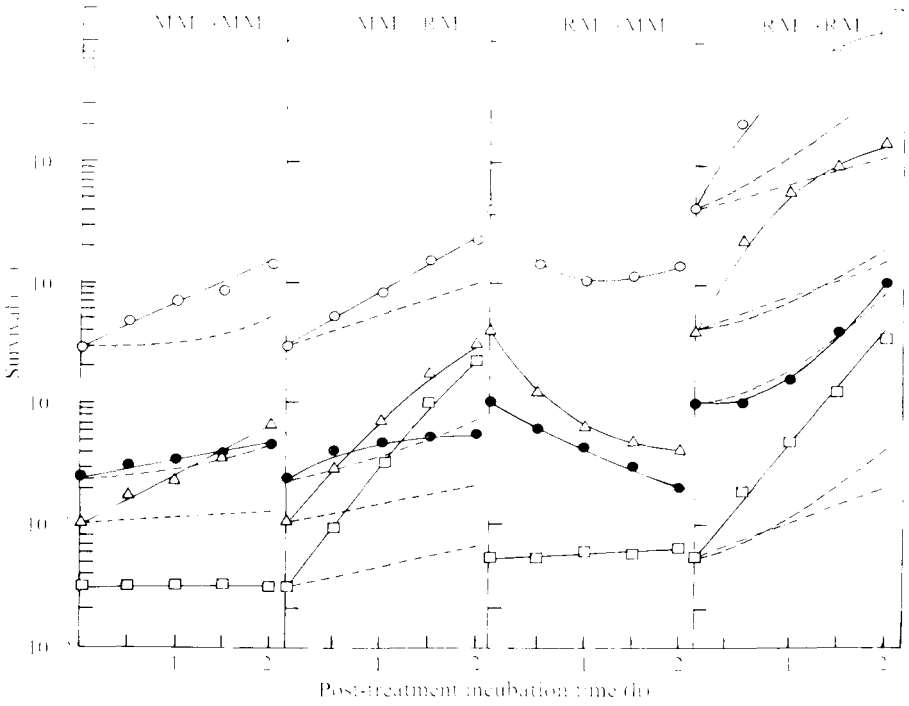

Fig. 2. Influence of pre- and post-treatment liquid media on the recovery of bacteria heated at 52 °C for 30 min: ○, wild type; ●, *lexA*; △, *recA*; □, *polA*. ○---○, Increase in the number of unheated bacteria under similar conditions; ○ - - - ○, viable count of heated bacteria in the presence of chloramphenicol.

Table 2. *Effect of metabolic inhibitors on the liquid holding recovery of heated* AB1157

Bacteria	Incubation medium	Time (min)	Viable units ml⁻¹ on different plating media: RM	RM + NaCl (2 %)	RM + mitomycin C
Unheated AB1157	RM	0	$2{\cdot}0 \times 10^{7}$	$2{\cdot}2 \times 10^{7}$	$2{\cdot}0 \times 10^{7}$
	RM	120	$1{\cdot}4 \times 10^{7}$	ND	ND
	RM + Rif	120	$1{\cdot}2 \times 10^{7}$	ND	ND
	RM + Cam	120	$2{\cdot}0 \times 10^{7}$	ND	ND
Heated AB1157 (30 min, 52 °C)	RM	0	$4{\cdot}3 \times 10^{5}$	$3{\cdot}5 \times 10^{4}$	$3{\cdot}5 \times 10^{4}$
	RM	120	$6{\cdot}5 \times 10^{6}$	$1{\cdot}2 \times 10^{6}$	$4{\cdot}0 \times 10^{6}$
	RM + Rif	120	$4{\cdot}5 \times 10^{4}$	$2{\cdot}2 \times 10^{5}$	$3{\cdot}0 \times 10^{4}$
	RM + Cam	120	$1{\cdot}2 \times 10^{6}$	$2{\cdot}5 \times 10^{5}$	$3{\cdot}3 \times 10^{5}$

ND, not done; Cam, chloramphenicol; Rif, rifampicin.

Heated bacteria were more sensitive to u.v. than unheated bacteria, but regained normal u.v. resistance during the 2 h incubation in recovery medium (Fig. 3). Similarly, heated bacteria were more sensitive to mitomycin C and to plating on high salt medium, but incubation in recovery medium resulted in normal mitomycin C resistance and high salt tolerance (Table 2).

Effect of chloramphenicol and rifampicin on recovery

The presence of chloramphenicol in the recovery medium during 2 h incubation of heated bacteria inhibited the recovery of viability. However, some recovery did occur even in the

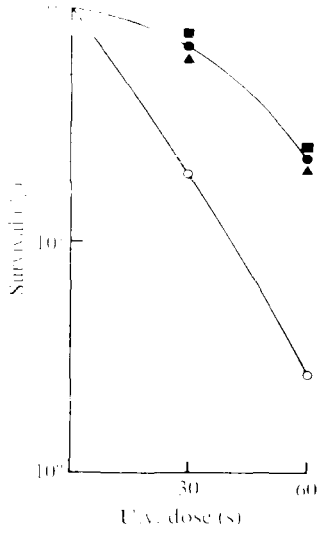

Fig. 3

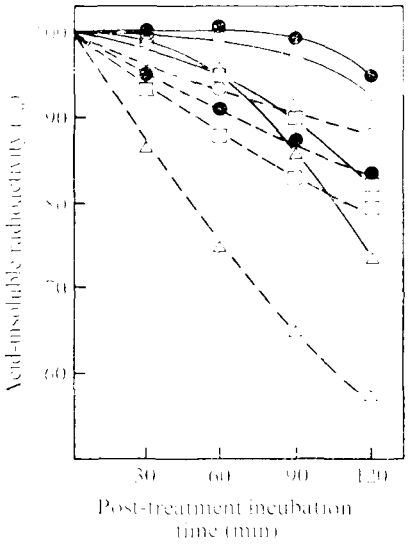

Fig. 4

Fig. 3. U.v. sensitivity of strain AB1157. ○, Bacteria heated at 52 °C for 30 min before exposure to u.v.; ■, unheated bacteria exposed to u.v.; ●, heated bacteria exposed to u.v. after shaking in recovery medium for 2 h; ▲, heated bacteria exposed to u.v. after shaking in recovery medium containing chloramphenicol.

Fig. 4. DNA degradation in heated (——; 52 °C for 30 min) and unheated (– – –) bacteria during post-treatment incubation in recovery medium: ○, wild type; ●, *lex*; △, *recA*; □, *polA*.

presence of chloramphenicol (Table 2, Fig. 2*d*). Chloramphenicol interfered to a large extent with the recovery of salt tolerance and mitomycin C resistance (Table 2), but had no effect on the recovery of u.v. resistance (Fig. 3).

Rifampicin totally inhibited the repair of heated bacteria in recovery medium. The degree of inhibition of salt tolerance was the same as that by chloramphenicol.

DNA degradation

The initial radioactivity in the acid-insoluble fractions of heated and unheated bacteria was less than 100% and varied from strain to strain, e.g. for unheated wild type, *recA*, *lex* and *polA* bacteria, the values were 90, 65, 80 and 82%, respectively, and the corresponding values for heated bacteria were 95, 70, 80 and 75%. So, for convenience, the initial counts were normalized to 100% and the rest of the results were calculated accordingly. DNA degradation was significantly inhibited in heated bacteria of each strain (Fig. 4).

DISCUSSION

Bridges *et al.* (1969) found that 'reckless' and 'cautious' recombination-deficient strains of *E. coli* B were sensitive to mild heating whereas the *exr* (*lex*) mutation had little or no effect except when combined with a *lon* or *her* mutation. The heat sensitivity of *polA*⁻ and *polA* strains was identical (Sedgwick & Bridges, 1972) and the authors explained this as being due to the lack of endonucleolytic incision activity. Part of our data on the apparent involvement of known loci on heat sensitivity do not agree with these results. We found that besides the *recA* strain, *polA* and *lex* strains were also sensitive to heat whereas the 'cautious' strain *recB* was as resistant as the wild type. The 'cautious' recombination-deficient strain used by Bridges *et al.* (1969) was later shown to have a *resA* mutation (Kato & Kondo,

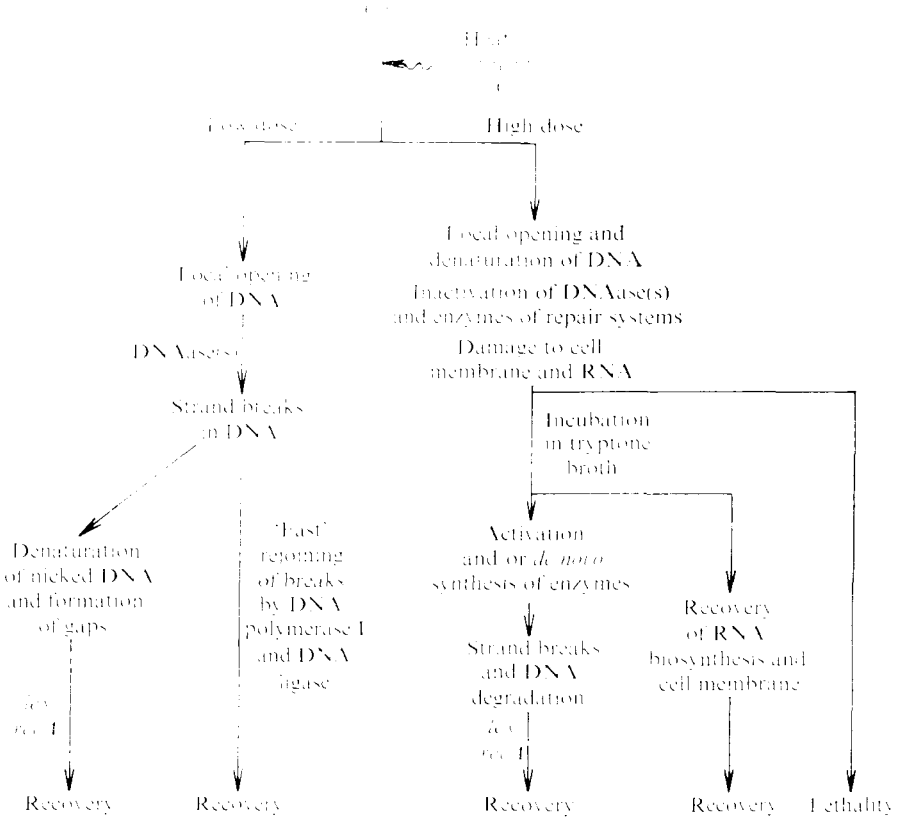

Fig. 5. Scheme for heat lesions and their mode of recovery.

1970), which is an allele of *polA*. This might explain why the 'cautious' recombination-deficient strain of Bridges *et al.* (1969) was sensitive to heat killing.

Survival was enhanced when the heated bacteria were held in tryptone broth as compared to direct plating on tryptone agar. The effect of 'liquid holding' on the recovery of u.v.-irradiated *E. coli* in MM is well documented (Roberts & Aldous, 1949; Ganesan & Smith, 1968). Contrary to the results with u.v., the liquid holding recovery of heated bacteria was poor in MM compared with recovery in RM.

Recovery of viability was considerably inhibited by rifampicin and chloramphenicol, suggesting a possible role for RNA and protein synthesis in recovery. *Salmonella typhimurium* also required both for recovery from thermal injury (Tomlins & Ordal, 1971), although other bacteria do not require protein synthesis (Clark *et al.*, 1968; Miller & Ordal, 1972; Gray *et al.*, 1973).

DNA degradation was inhibited by heat treatment, probably due to inhibition of DNAases. Chapmann & Pollard (1969) reported inhibition of DNA degradation when *E. coli* was heated at temperatures greater than 45 C prior to γ-irradiation. Heated bacteria were more sensitive to u.v. and to plating on high salt medium, and during liquid holding normal u.v. resistance and salt tolerance were regained. These data suggest inactivation of radiation repair enzymes and damage to the cell membrane by heat and their subsequent recovery. The recovery of normal u.v. resistance in the presence of chloramphenicol (Fig. 3) suggests that *de novo* synthesis of repair enzymes was not required and under favourable conditions, provided by tryptone broth, renaturation of the repair enzymes might be involved. Chloramphenicol inhibited the recovery of mitomycin C resistance and salt tolerance (Table 2) which suggests that repair of the cell membrane requires *de novo* protein synthesis. Thus,

the failure to achieve normal mitomycin C resistance in the presence of chloramphenicol could be due to increased permeability of mitomycin C. The recovery of heated u.v. resistance in the presence of chloramphenicol (Fig. 3) but inhibition of recovery of viable bacteria in recovery medium (Fig. 2*d*) suggest that the two phenomena may be independent of each other.

The repair of certain heat lesions occurred during liquid holding and not on agar plates of the same medium composition. We suggest that lesions in protein and membrane are repaired in the liquid medium but inhibited on agar plates. A similar result was reported by Roberts & Aldous (1949) who tested the inhibitory effect of agar on recovery of u.v.-irradiated *E. coli*. Recovery was obtained in liquid medium but was inhibited on agar medium and they attributed this effect to a typical diffusion which was not possible on the solid medium.

We propose the following explanation for the heat lesions and their mode of recovery in *E. coli* (Fig. 5). Short periods of heat treatment (low dose) cause local opening of DNA at various points (Inman, 1966). These specific regions are attacked by DNAase(s) (Goldmark & Linn, 1970; Sedgwick & Bridges, 1972) resulting in nicked DNA. A few breaks are repaired by DNA polymerase I and ligase (Town, Smith & Kaplan, 1971, 1972; Srivastava, 1974), while the remaining breaks require functional *lex* and *recA* genes. Thus, at low doses only the lesions in DNA are important and are repairable (Woodcock & Grigg, 1972), but after longer periods of heating, damage to RNA and protein becomes effective. This involves inactivation of repair enzymes and DNAase(s), and damage to the cell membrane. Recovery at higher doses would eventually require the synthesis of membrant and activation and/or *de novo* synthesis of enzymes. Post-treatment incubation in tryptone broth, but not on tryptone agar, brings about resumption of enzyme activities and cell membrane recovery, as shown by the increase in u.v. and mitomycin C resistance and high salt tolerance.

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Rapid Method for the Purification of Bacteriophage Lambda by Gel Filtration*

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A method for the purification of phage λ by gel filtration has been described. The crude lysate of phage is subjected to nucleases and polyethylene glycol treatments prior to loading on a Sephadex G-100 column. The purified phage is eluted in the void volume.

BACTERIOPHAGES are being used in genetical, biochemical and radiobiological studies. The stocks of phage λ obtained by lytic growth in a sensitive host contain constituents of growth medium, bacterial RNA and DNA, and other cellular components. A purified phage stock is, therefore, essential for biochemical and radiobiological work. The popular method of purification of phage λ involves caesium chloride equilibrium centrifugation[1-3], followed by removal of caesium chloride from the phage fraction. Though the method is simple, it is very expensive and not suitable in countries like India in view of the cost and maintenance of ultracentrifuges, nitrocellulose tubes etc. We have therefore developed a technique of phage purification by gel filtration which is efficient for bacteriophage λ and is relatively inexpensive and rapid.

The crude lysate of phage was made either by confluent lysis of *Escherichia coli* K-12 on nutrient agar plates or by lysis of infected bacteria in shaken tryptone broth[4]. Heavy particles were removed by centrifugaion at 5,000 rpm at 4°C for 15 min.

The crude stocks were successively treated with bovine pancreatic RNase (10 μg/ml) for 30 min at 42°C and with pancreatic DNase (10 μg/ml) in presence of 0.01 *M* Mg^{2+} for 30 min at 37°C. During nuclease treatment the lysate was gently shaken. The lysate was then centrifuged at 5000 rpm for 10 min to remove fibrous debris.

The volume of nuclease treated phage lysate was measured and crystals of sodium chloride were added to give final concentration of 0.5*M*. Polyethylene glycol (10% wt/vol.; PEG; type 6000; Belgolabo, Belgium) was added to the lysate which was then gently shaken for 3 hr on a magnetic stirrer at 4°C. PEG-treated phage lysate looked slightly opalescent. This was centrifuged in cold at 10000 rpm for 20 min. The supernatant was gently poured off and the pellet (adhered material) containing phage particles was suspended in 2 ml tris-Mg buffer (0.01*M*, *p*H 8). Thus a 100 ml lysate had been reduced to 2 ml. The 2 ml buffer containing phage was centrifuged once at 2000 rpm for 5 min to remove heavy insoluble materials.

Purification of phage was carried out on Sephadex G-100 (Pharmacia fine chemicals, USA) column at 10 to 20°C. The column specifications are given below :

Diam. of column	1.4 cm	Bed vol.	38.3 ml
Mass of dry gel used (Approx.)	2.4 g	Void vol.	13.8 ml
Bed length	25.0 cm	Flow rate	0.3 - 0.35 ml/min.

Blue dextran was used to determine the void volume of the column. The equilibration of the column and elution of phage were invariably done in tris-Mg buffer (0.01*M*, *p*H 8.0). Only 2 ml phage stocks were applied on the column. Fraction of

TABLE 1 — PURIFICATION DATA OF λ *vir* APPLIED ON SEPHADEX COLUMN

Steps	Total applied protein/DNA/ RNA in mg	Total eluted protein/DNA/ RNA in phage peak (mg)	Phage titre of applied stock
Crude lysate	8/0.6/0.45	0.2/0.34/0.24	7.4×10^9
Nuclease treated	4.5/0.23/0.20	0.15/0.08/0.12	7.3×10^9
Nuclease-PEG treated	0.3/0.09/0.08	0.07/0.03/0.035	2.8×10^{11}

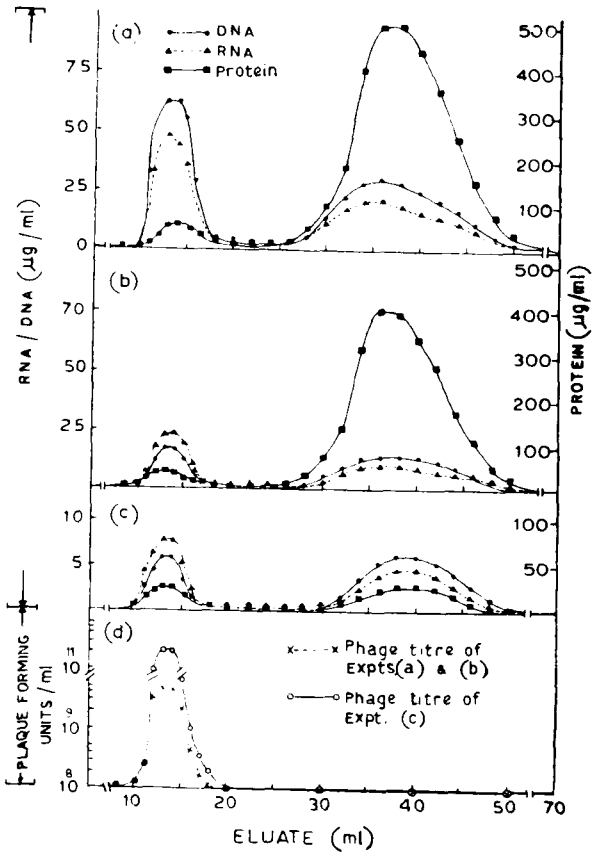

Fig. 1 — Gel chromatography of λ*vir* after various steps : (a) Crude stock; (b) Nuclease treated stock; (c) Nuclease-PEG treated stock; (d) Elution of phage particles

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2 ml each were collected, but in the vicinity of phage peak, the size of the fractions was reduced to 1 ml.

Estimations of DNA, RNA and proteins in each fraction were done according to the methods described by Burton[5], Ceriotti[6] and Lowry *et al.*[7] using ealf thymus DNA, purified yeast RNA and bovine serum albumin as standards respectively. Phage titre was determined by plaque forming ability on *E. coli* K-12.

According to the technique described above, the crude stocks of *λvir*, *λCI*857 and *λb*2 were purified. As shown in Fig. 1d, the phage particles were eluted in the void volume, whereas most of the impurities which include bacterial DNA, RNA, protein and other molecules of low molecular weight were eluted in the fractions quite different from those of the phage (Fig. 1). The separation volume was approximately 20 ml. It can be seen from Table 1 that a purified phage stock of high degree was obtained by nuclease-PEG treatment followed by gel filtration on Sephadex G-100 column. Invariably 98% of the phage titre was recovered after purification on the column. Since the total phage particles were collected into 4 identical fractions (Fig. 1) the stock was eventually diluted 4 times. Owing to the low molecular weight of nucleases there is no possibility of their contamination in phage fraction.

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